**Electrospun curcumin-loaded protein nanofiber mats as active/bioactive
coatings for food packaging applications**

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ABSTRACT

In this study, curcumin was encapsulated within electrospun protein (i.e. gelatin and zein) fibers to generate bioactive coatings for food packaging. Additionally, a green tea extract (GTE) was also incorporated within the formulations to evaluate its impact on the stability, protective ability, and release properties of the curcumin-loaded fibers.

Due to the poor solubility of curcumin in aqueous media, a strategy based on its incorporation through liposomes was developed, allowing to successfully incorporating curcumin into gelatin fibers. Very high encapsulation efficiencies were attained for both zein and gelatin, with the former showing an enhanced protection effect and a more limited and slower release of the curcumin in hydrophobic food simulants. The addition of GTE resulted in strong interactions being established with the proteins and, in the particular case of gelatin, it improved the protective effect and slowed down the curcumin release from the fibers, although it did not prevent their collapse in water.

The results showed that while the developed gelatin coatings showed a promising release behavior in contact with fatty food simulants, zein-based coatings would be more adequate for packaging of high water content food products.

Keywords: Electrospinning; curcumin; SAXS/WAXS; FTIR; thermal stability; controlled release.

Chemical compounds studied in this article: Curcumin (PubChem CID: 969516).

1. INTRODUCTION

The sector of food packaging has tremendously evolved in the last decades in response to the growing challenges imposed by our modern society. Nowadays, packaging materials are no longer regarded as mere storage containers for food products, but they are also expected to actively prolong their shelf-life, guarantee their safety and preserve or even improve their quality (Realini & Marcos, 2014; Vanderroost, Ragaert, Devlieghere, & De Meulenaer, 2014). Accordingly, active food packaging systems are designed to play an active role in food preservation beyond their basic function as a barrier between foodstuffs and the external environment, either by scavenging undesirable substances or by releasing preserving agents (Ozdemir & Floros; Vilela et al., 2017). Furthermore, a novel concept of bioactive or functional packaging has recently emerged and defined as a series of packaging technologies or approaches intended to aid in the production of functional foods, that is, to enhance the impact of food on the consumer's health (López-Rubio, 2011).

One approach for the design of bioactive or functional food packaging materials is to incorporate beneficial bioactive ingredients within them, which must be then released towards the food products in a controlled manner during their shelf-life. Since most bioactive compounds are highly sensitive, and may be degraded during the usual thermal processing of plastics and bioplastics, their direct incorporation within the bulk packaging material is not feasible in practice (Fabra, López-Rubio, & Lagaron, 2016). A plausible alternative is to encapsulate them and apply them as coatings on the food contact surface of the pre-formed packaging container. In this context, electrohydrodynamic processing is a promising technology which has recently attracted increased interest both for the encapsulation of functional ingredients (Gómez-Mascaraque, Hernández-Rojas, et al., 2017; Gómez-Mascaraque, Lagarón, & López-Rubio, 2015; Gómez-Mascaraque & López-Rubio, 2016) and for the development of active coatings for food packaging (Castro-Mayorga, Fabra,

Cabedo, & Lagaron, 2016; Chalco-Sandoval, Fabra, López-Rubio, & Lagaron, 2017; Dumitriu, Mitchell, Davis, & Vasile, 2017). It is a simple, efficient, versatile, cost-effective and scalable technology to produce dry nano- and microencapsulation structures in the form of fiber mats (electrospinning) or particulate powders (electrospraying), by subjecting a polymeric fluid to an external high-voltage electric field (Bhushani & Anandharamakrishnan, 2014; Echegoyen, Fabra, Castro-Mayorga, Cherpinski, & Lagaron, 2017). Due to its great potential, a number of review papers about the electrohydrodynamic processing technology (Bhattacharjee & Rutledge, 2017; Xie, Jiang, Davoodi, Srinivasan, & Wang, 2015; Zong et al., 2018) and its specific applications in the food sector (Alehosseini, Ghorani, Sarabi-Jamab, & Tucker, 2017; Bhushani et al., 2014; Echegoyen et al., 2017; Mendes, Stephansen, & Chronakis, 2017) have been very recently published, to which the readers are directed for further information about its basic principles and functioning.

Some of the advantages of electrohydrodynamic processing as an encapsulation technique include the feasibility of obtaining dry, food-grade, nanostructured materials in a one-step process at mild temperature conditions (suitable for labile ingredients) with high encapsulation efficiencies. Moreover, by changing the solution properties and process parameters, the morphology of the obtained materials can be varied (Alehosseini et al., 2017; Echegoyen et al., 2017; Kriegel, Arrechi, Kit, McClements, & Weiss, 2008), allowing, for instance, the production of continuous layers in the form of fiber mats. Electrospun nanofibers present particular benefits for the production of food packaging coatings, such as higher crack resistance as compared to conventional films, absence of negative impact in the flexibility and mechanical properties of the packaging material to be coated and their good adhesion properties due to their large surface to volume ratio (Fabra, Busolo, Lopez-Rubio, & Lagaron, 2013). For these reasons, electrospinning has already been effectively used in the food packaging area to generate antimicrobial coatings (Castro-Mayorga et al., 2017), high

85 barrier structures (Fabra, Busolo, et al., 2013; Fabra, Lopez-Rubio, & Lagaron, 2013),
86 adhesive interlayers (Fabra, López-Rubio, & Lagaron, 2014), packaging materials with
87 temperature buffering capacity (Chalco-Sandoval et al., 2017; Chalco-Sandoval, Fabra,
88 López-Rubio, & Lagaron, 2014) and, very recently, functionalized antioxidant coatings based
89 on α -tocopherol (Dumitriu et al., 2017; Fabra et al., 2016).

90 Regarding the materials to be used as coatings for food packaging, biopolymers are very
91 advantageous due to their availability, non-toxicity, biodegradability and renewability, and
92 their unique properties (Mellinas et al., 2016). Among them, proteins have been recognized
93 for their structural stability, which facilitates the formation of edible coatings capable of
94 retaining a given shape (Fabra, Busolo, et al., 2013). Furthermore, they have been described
95 to strongly interact with natural polyphenols through hydrophobic interactions and hydrogen
96 bonding (Jakobek, 2015; Siebert, Troukhanova, & Lynn, 1996), implying that the stability of
97 electrospun protein fibers could be further improved by the addition of polyphenols as natural
98 crosslinkers for proteins (Anvari & Chung, 2016). In particular, zein and gelatin are two
99 proteins whose ability to form electrospun fibers has been already demonstrated (Lu et al.,
100 2015; Yao et al., 2018). In addition, both proteins have previously shown their potential to
101 enhance the stability of sensitive bioactive ingredients encapsulated within them through
102 electrohydrodynamic processing (Gómez-Mascaraque et al., 2015; Gómez-Mascaraque,
103 Perez-Masiá, González-Barrio, Periago, & López-Rubio, 2017). For these reasons, both
104 proteins were selected in this work as matrices to generate functional coatings for food
105 packaging. On the other hand, curcumin was selected as a model bioactive compound to be
106 incorporated within the bioactive coatings. Curcumin is a natural, bioactive ingredient which
107 has been widely recognized for its health-promoting properties, including antioxidant, anti-
108 inflammatory, and antimutagenic activities (Araiza-Calahorra, Akhtar, & Sarkar, 2018; Chen
109 et al., 2009). Moreover, it has been acknowledged for its antimicrobial properties, which

together with its antioxidant capacity makes it a promising food preservative (Gómez-Estaca, Balaguer, López-Carballo, Gavara, & Hernández-Muñoz, 2017). Therefore, it is an excellent candidate for the design of simultaneously active and bioactive food packaging coatings. Accordingly, the aim of this work was the development of curcumin-loaded coatings through electrospinning which could serve as functional food contact layers for active and bioactive food packaging. Gelatin and zein were used as vehicles for the incorporation of the antioxidant, and the produced fiber mats were characterized and compared in terms of their stabilization effect on the bioactive molecule, as well as their release behavior in two different food simulants. A polyphenol-rich green tea extract was also incorporated within the formulations in order to assess the impact of its interactions with the proteins on the stability of the curcumin-loaded fibers, their protective ability and their release properties.

2. MATERIALS AND METHODS

2.1. Materials

Type A Gelatin from porcine skin, with reported gel strength of 180 g Bloom, was obtained from Gelita (Germany). Zein from corn (grade Z3625), with reported molecular weight of 22–24 kDa, was purchased from Sigma-Aldrich (Spain). Pure phosphatidylcholine (98% ± 4%) stabilized with 0.1% ascorbyl palmitate, under the commercial name of Phospholipon® 90G, was kindly donated by Phospholipid GmbH (Germany). Curcumin (65%) was procured from Sigma-Aldrich (China), soybean oil (SBO) and phosphate buffered saline system (PBS, pH = 7.4) were obtained from Sigma-Aldrich (Spain). A green tea extract (GTE) with high oxygen radical absorbance capacity (ORAC) was kindly donated by Naturex (Avignon, France). 96% (v/v) acetic acid was supplied by Scharlab (Spain). Absolute ethanol (>99.9%) was purchased from VWR (UK).

2.2. Preparation of the protein solutions

Gelatin solutions (20% w/w) were prepared by dissolving the biopolymer in acetic acid 20% (v/v) under magnetic agitation, as previously described in Gómez-Mascaraque et al. (2015). Since curcumin is not soluble in aqueous media, when it was incorporated within the gelatin formulations, liposomal curcumin dispersions were first prepared as described in Section 2.3, and gelatin (20% w/w) was then dissolved directly in these liposomal curcumin dispersions under magnetic agitation and at room temperature.

Zein solutions (20% w/w) were prepared by dissolving the protein in ethanol 80% (v/v) under magnetic agitation, as previously described (Li, Lim, & Kakuda, 2009). When curcumin was incorporated, it was added to the zein solutions at room temperature under magnetic stirring, at a concentration of 0.16% w/w of the total solids content. This concentration was adjusted to obtain the same curcumin content in both gelatin and zein fibers (cf. Section 2.3).

To evaluate the impact of adding polyphenols to the formulations on the performance of the obtained coatings, these were also produced adding 10% (w/w) GTE with respect to the proteins (Gómez-Mascaraque, Hernández-Rojas, et al., 2017) to the aforementioned gelatin and zein formulations.

2.3. Liposomes production

Liposomes were prepared using the ethanol injection method based on the protocol described in Gómez-Mascaraque, Casagrande Sipoli, de La Torre, and López-Rubio (2017), with some modifications. Briefly, phosphatidylcholine was dispersed in absolute ethanol at a concentration of 80 g/L and added dropwise to acetic acid 20% (v/v) in a volumetric ratio of 10% under continuous stirring at 11000 rpm using a high-speed homogenizer (MICCRA D-9,

Germany) equipped with a VARIO (DS-20/PG SMIR) dispersing element (stator diameter of 20 mm). The agitation was maintained for 5 min after the addition was completed.

Curcumin-loaded liposomes were produced by adding 5% (w/w) of curcumin (with respect to the lipid) to the ethanolic phosphatidylcholine solution prior to the formation of the liposome dispersions in acetic acid 20% (v/v).

Liposome dispersions in water, similar to those produced in Gómez-Mascaraque, Casagrande Sipoli, et al. (2017), were also produced for comparison purposes, by replacing the acetic acid 20% (v/v) with MilliQ water.

2.4. Characterization of the liposomes

2.4.1. Size distribution

The size distribution of the prepared liposomes was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS equipment (Malvern Instruments Corp., UK), according to the method described in Casagrande Sipoli, Santana, Shimojo, Azzoni & de la Torre (2015). For this purpose, the liposome dispersions were previously diluted to a phosphatidylcholine concentration of 20 µg/ml with acetic acid 20% (v/v) or MilliQ water, respectively. All measurements were made in triplicates and intensity-weighted results are reported.

2.4.2. Transmission electron microscopy (TEM)

One drop (8 µL) of the liposome dispersion in water (diluted at a concentration of a 0.001%) was allowed to dry on a carbon coated grid (200 mesh) and the sample was stained with uranyl acetate. TEM was performed using a JEOL 1010 (Tokyo, Japan) microscope at an accelerating voltage of 80 kV.

2.4.3. Small angle X-ray scattering (SAXS)

The liposomes were structurally characterized at the nanoscale level by means of SAXS experiments conducted 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. (2018). The liposomal dispersions were placed in sealed 2 mm quartz capillaries (Hilgenburg GmbH, Germany). 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.

2.5. Production of electrospun mats

The protein solutions were introduced in a 5 mL plastic syringe and processed using an electrospinning apparatus assembled in house in a horizontal configuration, equipped with a variable high-voltage 0-30 kV power supply (Acopian, USA) and a syringe pump (KD Scientific, USA). For this purpose, the feed solutions were pumped through a stainless-steel needle with an inner diameter of 0.9 mm, which was connected to the syringe through a PTFE tube and was placed perpendicularly to the stainless-steel plate used as collector, at a distance of 10 cm. The flow rate and applied voltage were optimized for each protein, and were varied in the ranges between 0.15-1.5 mL/h and 12 kV, respectively.

2.6. Characterization of the electrospun coatings

2.6.1. Morphological characterization

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 20 kV and a working distance of ~ 10 mm, as previously described in (Gómez-Mascaraque, Sanchez, & López-Rubio, 2016), after sputter-coating the samples with a gold-palladium mixture under vacuum.

208

209 **2.6.2. Fourier transform infrared (FT-IR) analysis**

210 Electrospayed fibers and powdery materials were subjected to FTIR spectroscopy (Cary 630,
211 Agilent, USA) and analyzed in transmission mode in a range of 650 to 4000 cm^{-1} . The lipids'
212 spectrum was obtained without further processing in ATR mode. Each recorded spectrum
213 was determined from an average of 16 scans at 4 cm^{-1} resolution (Gómez-Mascaraque,
214 Casagrande Sipoli, et al., 2017).

215

216 **2.6.3. Thermogravimetric analysis (TGA)**

217 Thermogravimetric analysis (TGA) was performed with a TG-STDA Mettler Toledo model
218 TGA/STDA851e/LF/1600 analyzer. The samples (ca. 10 mg) were heated from 25 °C to 600
219 °C at a heating rate of 10 °C/min under dynamic nitrogen flow. Derivative thermogravimetric
220 (DTG) curves express the weight loss rate (dm/dT) as a function of temperature.

221

222 **2.6.4. Small angle X-ray scattering (SAXS)**

223 In order to assess the structural changes undergone by the liposomes when being subjected to
224 the electrospinning process, the gelatin solutions with liposomes and the generated
225 electrospun fibers were characterized by means of SAXS experiments following the method
226 described in Section 2.4.2. In this case, the fibers were placed in their native dry state and run
227 using a perforated sample holder.

228

229 **2.7. Curcumin encapsulation efficiency**

230 The total amount of curcumin effectively incorporated within the fiber mats was estimated by
231 UV-Vis spectroscopy according to a protocol adapted from Gómez-Mascaraque, Casagrande
232 Sipoli, et al. (2017). For this purpose, gelatin fibers (40 mg/mL) were first dissolved in acetic

acid 20% (v/v) and the obtained dispersions were subsequently diluted 4-fold in absolute ethanol to completely dissolve the phospholipids and the curcumin. On the other hand, zein fibers (20 mg/mL) were directly dissolved in ethanol 80% (v/v), solvent in which both the protein and curcumin were soluble. In both cases, the absorbance of the resulting solutions was measured at 427 nm using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, USA). Calibration curves for curcumin quantification were previously obtained in both solvent mixtures ($R^2 > 0.995$), and the contribution of zein to the absorbance at 427 nm was taken into account. The encapsulation efficiency (EE) was then calculated according to Eq. (1). Three independent replicates of each sample were analyzed.

$$EE (\%) = \frac{\text{Total curcumin content in the fibres}}{\text{Theoretical curcumin content in the fibres}} \times 100 \quad (\text{Eq. 1})$$

2.8. Curcumin degradation assays

In order to assess the stabilization effect of the protein fibers on curcumin, degradation assays were performed in PBS (pH = 7.4) following a protocol adapted from (Gómez-Mascaraque, Casagrande Sipoli, et al., 2017). Briefly, curcumin-loaded protein fibers were dispersed in PBS (pH = 7.4) to achieve theoretical curcumin concentrations of 0.01 mg/ml. Similarly, free curcumin was dissolved in PBS by first preparing a 1 mg/ml stock solution in absolute ethanol and subsequently diluting it 100-fold in PBS. After selected time intervals, the samples were further diluted 4-fold with absolute ethanol (for free curcumin and zein fibers) or acetic acid (for gelatin fibers) in order to dissolve the encapsulation structures and release their curcumin contents. The absorbance of the final solutions was then measured at 427 nm as described in section 2.7.

2.9. *In-vitro* release assays

The release of curcumin from the protein fibers was assessed in two different food simulants following a method adapted from Atay et al. (2018). Briefly, the fiber mats were immersed in the release media at a concentration of 30 mg/mL and the absorbance of the supernatant at 427 nm was measured after selected time intervals using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA), after calibration ($R^2>0.998$). The samples were gently shaken to homogenize them before analysis of the supernatant. Ethanol 50% and a vegetable oil (soy bean oil) were selected as food simulants, according to the Commission Regulation 10/2011 EU on plastic materials and articles intended to come into contact with food (10/2011/EC). Experiments were performed at room temperature ($\sim 25^\circ\text{C}$) in independent duplicates.

2.10. Fiber stability assessment

Stability of the fiber mat samples was assessed based on a protocol adapted from Kiechel and Schauer (2013). Briefly, samples were electrospun on aluminum foil as a support and 15 mm $\times$ 70 mm pieces were cut. Each piece was immersed in a separate falcon tube containing 40 mL of water. After selected time intervals, the protein coatings were removed from the falcons and dried in a desiccator equilibrated at 0% relative humidity. The surface morphology of the dry samples was then analyzed by SEM as described in Section 2.6.1.

2.11. Statistical analysis

The statistical analysis of experimental data was performed using IBM SPSS Statistics software (version 23, IBM Corp., USA). One-way analysis of variance (ANOVA) was done to determine the significant differences between sample means at a significance level of $\alpha=0.05$. Mean comparisons were performed by the Duncan's Test. All experiments were performed in triplicate, and data are presented as mean $\pm$ standard deviation (SD) values.

3. RESULTS AND DISCUSSION

3.1. Curcumin-loaded phosphatidylcholine liposomes

Curcumin is poorly soluble in aqueous media. Therefore, although it could be readily dissolved in the ethanolic zein solution prior to electrospinning, an effective strategy to disperse curcumin in the aqueous gelatin solution had to be developed. Recently, aqueous liposome dispersions have been proposed as a promising vehicle for the incorporation of curcumin within electrosprayed protein microencapsulation structures (Gómez-Mascaraque, Casagrande Sipoli, et al., 2017). On the other hand, gelatin cannot be electrospun at room temperature when dissolved in water, so diluted acetic acid is usually employed as solvent to produce non-toxic gelatin nano- or microstructures by electrohydrodynamic processing (Gómez-Mascaraque et al., 2015).

Accordingly, phosphatidylcholine liposomes were prepared in 20% v/v acetic acid through the ethanol injection method, and their size was compared to that of standard phosphatidylcholine liposomes prepared in purified water under the same conditions. Although the pH at which the liposomes are prepared has been previously shown to have an impact on their size (King & Marchbanks, 1982), to the best of our knowledge there is no published information about the effect of the presence of acetic acid on the size of phosphatidylcholine liposomes prepared by the ethanol injection method. Table 1 summarizes the average size and polydispersity index (PDI), intensity weighted, of the prepared liposomes, estimated by DLS measurements. Interestingly, the results suggest that the hydrodynamic diameter of the obtained liposomes was significantly reduced when they were prepared in diluted acetic acid ($P < 0.05$).

Table 1. Average size and polydispersity index (PDI) of the prepared phosphatidylcholine liposomes*

Sample description	Average hydrodynamic diameter (nm)	PDI
Unloaded liposomes in water	741±27 ^a	0.28±0.01
Unloaded liposomes in 20% acetic acid	197±18 ^b	0.38±0.03
Curcumin-loaded liposomes in 20% acetic acid	154±2 ^c	0.26±0.01

*Different lower case letters in superscript indicate significant difference ($p < 0.05$).

To confirm this, the liposome dispersions in water were also characterized by TEM and representative images are shown in Figure 1. Although the average liposome diameter measured from the images was significantly smaller than that determined by DLS, i.e. 104 ± 75 nm, from the images it was evident that the size of the liposomes presented a large polydispersity and some agglomerates with much larger size, formed by the association of several liposomes, were also detected in the sample (cf. Figure 1B). The discrepancy between the average liposome diameter values estimated from DLS and TEM is most likely due to the great effect of larger particles in the diameter estimated by DLS. In that case, since the intensity of the light scattered is proportional to the diameter to the sixth power, the presence of a small amount of large particles can mask the actual diameter of the smaller particles in the sample (Plantz, 1984).

The size of the liposomes is a key factor impacting the electrohydrodynamic processing of protein dispersions, since too big liposomes cause a disturbance of the Taylor cone, which results in dripping of the suspensions (Gómez-Mascaraque, Llavata-Cabrero, Martínez-Sanz, Fabra, & López-Rubio, 2018). In fact, post-production treatments, such as ultrasonication, were required in our previous work in order to reduce the liposome sizes and achieve a stable electrospraying process (Gómez-Mascaraque, Casagrande Sipoli, et al., 2017). However, this step was not necessary when the liposomes were produced in 20% acetic acid, since,

apparently, liposomes with average hydrodynamic diameters lower than 200 nm were obtained, facilitating the subsequent electrospinning process (cf. Section 3.2).

Figure 1. TEM images from the liposome dispersions in water.

To obtain a more detailed structural characterization of the liposomal dispersions, SAXS experiments were performed, and the reduced data are displayed in Figure 2A. As observed, the scattering patterns were characterized by the appearance of several structural features, being the peak located at $q=0.030\text{--}0.031\text{ \AA}^{-1}$ the most evident feature for the three samples. Such peak corresponds to real distances of ca. 20.3 nm for the unloaded liposomes (in water and acetic acid), and ca. 20.7 nm for the liposomes loaded with curcumin. In addition, a peak located at approximately $q=0.099\text{ \AA}^{-1}$ was also visible in the SAXS pattern from the liposomes dispersed in water, although due to the noise arising from the incoherent scattering background within this region, it did not appear as a well-defined peak. This peak corresponds to a real distance of ca. 6.4 nm and, according to previous works (Sakuragi et al., 2011; Thompson, Hindmarsh, Haisman, Rades, & Singh, 2006; Winter, 2002), it may arise from the characteristic distance of the lipid bilayer membrane. In the case of the liposomes dispersed in acetic acid (both unloaded and loaded with curcumin), this peak was not clearly discerned. This might be due to a greater polydispersity in the size of the lipid bilayer when

using acetic acid instead of water as the solvent. It should be mentioned that the shape of the SAXS patterns, with very broad peaks which do not show second order reflections, is indicative of the presence of unilamellar or multivesicular liposomes (Thompson, et al., 2006). The increase in the scattering intensity observed for the liposomes loaded with curcumin indicates that the scattering length density contrast generated between the liposomes and the surrounding solvent was increased as a result of the incorporation within the liposomes of an additional component with higher scattering length density, such as the curcumin. As shown in Figure 2B, shoulder-like features, located within the region of $q=0.004\text{-}0.012\text{ \AA}^{-1}$, were also evident in the scattering patterns from all the samples. These features may arise from the spherical structure factor of the liposomes. The overall diameter of the liposomes, as estimated from the DLS experiments, was close to the lower size range limit covered by the SAXS experiments and, thus, only the weaker higher order reflections were visible in the patterns.

Figure 2. (A) SAXS patterns of the unloaded phosphatidylcholine liposome dispersions in water and acetic acid and the liposomes loaded with curcumin, dispersed in acetic acid. (B) SAXS Kratky plot highlighting the appearance of shoulder-like features (pointed out by arrows).

3.2.Characterization of the electrospun coatings

3.2.1. Morphological characterization

Curcumin-loaded nanofiber mats were produced from the protein solutions through electrospinning. For this purpose, curcumin was directly dissolved in the zein solutions, and gelatin was dissolved in the liposomal curcumin suspensions previously prepared.

A preliminary optimization of the electrospinning parameters was conducted in order to select the best conditions for continuous fiber production. Figures 3A and 3B show representative SEM images of the obtained zein and gelatin fibers, respectively.

The morphology of the obtained coatings was similar to those previously reported for zein (Li, et al., 2009; Neoet al., 2013) and gelatin (Kwak, et al., 2017; Okutan, Terzi, & Altay, 2014) electrospun mats, showing an ultrathin fibrillar structure. While zein fibers showed their characteristic flat, ribbon-like morphology, the gelatin fibers exhibited a more rounded shape. These differences in morphology have been attributed to the tendency of zein to form unexpanded β -folds (Wang et al., 2016), in contrast to the triple helix structure of gelatin (Guo, Colby, Lusignan, & Whitesides, 2003). Gelatin also yielded more homogeneous fibers and with smaller average diameters (230 ± 38 nm) than zein (360 ± 146 nm). It is worth noting that, due to their small size, the presence of liposomes within the gelatin fibers did not alter their beadless morphology, which displayed a smooth surface.

Fig. 3 SEM images of curcumin-loaded zein (A) and gelatin (B) electrospun fibers prepared in the absence and in the presence (zein (C) and gelatin (D)) of green tea extract, together with their diameter distributions. Scale bars correspond to 5 μm .

Polyphenols have been described to act as natural cross-linkers for proteins (Anvari et al., 2016), since they can strongly interact with them through hydrophobic interactions and hydrogen bonding (Jakobek, 2015; Siebert et al., 1996). Therefore, GTE-containing electrospun fibers were also produced in order to assess the impact of co-encapsulating a polyphenol-rich extract on the stability of the curcumin-loaded fibers, their protective ability and their release properties. Figures 3C and 3D show SEM micrographs of the zein and gelatin fibers, respectively, prepared in the presence of GTE. In both cases, the fiber diameter increased upon addition of the extract (480 ± 209 nm for zein and 500 ± 93 nm for gelatin), presumably due to some interactions between protein and polyphenol, on one hand, and the expected increase of viscosity in the final solutions as a result of their higher solids content, on the other hand. An increase in the diameter of zein fibers had been previously observed upon addition of (-)-epigallocatechin gallate (Li et al., 2009), which is the most abundant polyphenol found in the GTE (Gómez-Mascaraque, Hernández-Rojas, et al., 2017). Nevertheless, the characteristic ribbon-like and rounded morphologies of zein and gelatin fibers, respectively, were preserved despite the addition of GTE.

3.2.2. Curcumin encapsulation efficiency

In order to estimate the amount of curcumin effectively incorporated within the developed coatings, the encapsulation efficiency of the different systems was calculated according to Eq. (1). The results are summarized in Table 2.

Table 2. Curcumin encapsulation efficiency (%) for the developed coatings

	Zein fibers	Gelatin fibers
Without GTE	87±10 ^{ns}	89±2 ^{ns}
With GTE	86±2 ^{ns}	86±4 ^{ns}

ns: Not significant

Both proteins yielded similar curcumin encapsulation efficiencies, despite the differences in the protocol for bioactive incorporation within the protein solutions (i.e. dissolution vs. entrapment within liposomes). These values were considerably high, and similar to those previously reported for the microencapsulation of curcumin within these proteins through electrohydrodynamic processing (Gomez-Estaca, Balaguer, Gavara, & Hernandez-Munoz, 2012; Gómez-Estaca et al., 2017; Gómez-Mascaraque, Casagrande Sipoli, et al., 2017), emphasizing the suitability of the electrospinning technique for the encapsulation of sensitive bioactive compounds. On the other hand, the addition of GTE to the formulations did not have an impact on the curcumin encapsulation efficiency of the systems, since no significant differences were observed upon incorporation of the extract.

3.2.3. SAXS/WAXS

To investigate the possible structural changes undergone by the liposomes when combining them with gelatin in the form of hydrated systems and in dry fibers, the SAXS patterns from the electrospinning solutions and the obtained fibers were collected and the results are shown in Figure 4. From the characterization of the gelatin solutions (cf. Figure 4A), it is evident that the incorporation of liposomes in the acetic acid prior to dissolving the gelatin, had a significant effect on the shape of the scattering patterns. The pure gelatin solution presented a very similar pattern to that previously reported for 1% (Gómez-Mascaraque et al., 2018) and 8% w/w (Atay et al., 2018) gelatin solutions in acetic acid, with a weak shoulder-like feature

appearing within the q range of ca. $0.003\text{--}0.008\text{ \AA}^{-1}$, which is characteristic from the size of gelatin aggregates formed in acetic acid due to intra- and intermolecular electrostatic interactions, and a broad shoulder at ca. $0.03\text{--}0.22\text{ \AA}^{-1}$, which is characteristic of the distance between adjacent polymeric chains (Atay et al., 2018). In the case of the gelatin solutions prepared in the presence of liposomes, the gelatin features were less defined and a narrow peak located at $0.1141\text{ \AA}^{-1}$, corresponding to a real distance of ca. 5.5 nm , was detected. Since it was not possible to detect the peak associated to the lipid bilayer membrane in the pure liposome dispersion in acetic acid, it is not possible to ascertain whether the peak detected when gelatin was added to the system arose from the bilayer structure, which was affected by the presence of gelatin, or from the formation of a uniform gelatin layer coating the surface of the liposomes. In any case, the results seem to suggest that when gelatin was added to the medium, it was able to interact with the surface of the liposomes.

Fig. 4 (A) SAXS patterns of the pure gelatin solution in acetic acid and the gelatin solution prepared in the liposomal dispersion in acetic. (B) SAXS patterns of the liposome dispersions (with and without gelatin) and the generated electrospun fibers.

Interestingly, as shown in Figure 4B, whereas the gelatin structure seemed to predominate in the scattering pattern from the solutions, after subjecting it to the electrospinning process and

obtaining dry fibers, the structure of the liposomes predominated in the scattering data. The scattering intensity of the electrospun fibers was higher than that of the liposomal dispersions, due to the increased scattering length density contrast generated between the liposomes and the surrounding air as compared with the contrast existing between the liposomes and the acetic acid. The shoulders arising from the spherical shape of the liposomes (pointed out by grey arrows in Figure 4B) were more prominent than in the native liposomal dispersion. Furthermore, the peak associated to the liposomes' core was still visible in the fibers and appearing exactly in the same position. On the other hand, a small peak located at ca. 0.0765 Å⁻¹, characteristic from a real distance of 8.2 nm, was visible. Taking into account that in the dry state the largest scattering length density contrast should exist between the air gaps and the polymeric components in the system, this distance should be characteristic from the overall thickness of the gelatin-coated lipidic membrane from the liposomes. An important implication from these results is that, in agreement with previous studies (Kaldybekov, Tonglairoum, Opanasopit, & Khutoryanskiy, 2018), the structure of liposomes is not only formed in the hydrated state, but it is also preserved when the solvent is removed from the system and therefore, the curcumin should be retained within these liposomal structures even when electrospinning fibers are generated.

3.2.4. FT-IR spectroscopy analysis

Figure 5 shows the FT-IR spectra of gelatin and zein fibers containing curcumin, both in the presence and in the absence of GTE, together with the spectrum of the commercial GTE. FT-IR spectra are presented in absorbance mode.

The characteristic bands of proteins, i.e. amide A or N-H stretching (3287 cm⁻¹), amide B or asymmetric stretching vibration of =C-H and -NH₃⁺ (3071 cm⁻¹ for gelatin and 3058 cm⁻¹ for zein), amide I or C=O stretching (1629 cm⁻¹ for gelatin and 1647 cm⁻¹ for zein), amide II or

482 N-H bending and stretching (1528 cm^{-1} for gelatin and 1532 cm^{-1} for zein), and amide III or
483 C-N stretching (1236 cm^{-1} for gelatin and 1239 cm^{-1} for zein) (Aewsiri, Benjakul,
484 Visessanguan, Wierenga, & Gruppen, 2010; Nagarajan, Benjakul, Prodpran, Songtipya, &
485 Nuthong, 2013) were present in all the fiber mats.

486 While no significant changes were observed in the FTIR spectra in the unloaded and
487 curcumin-containing fibers, GTE incorporation gave rise to several spectral changes as
488 described below. The spectrum of commercial GTE showed characteristic bands from tea
489 catechins at $\sim 1602\text{ cm}^{-1}$, which could be attributed to the aromatic ring quadrant, $\sim 1515\text{ cm}^{-1}$,
490 corresponding to the aromatic semicircle stretch, $\sim 1451\text{ cm}^{-1}$, from the aromatic stretch
491 (C=C), $\sim 1308\text{ cm}^{-1}$, ascribed to the deformation vibration of O-H groups from the aromatic
492 alcohol, and $\sim 1090\text{ cm}^{-1}$, corresponding to the aromatic rings stretch (cf. gray circles in Fig. 5)
493 (Gómez-Mascaraque et al., 2015; Robb, Geldart, Seelenbinder, & Brown, 2002).

494

Figure 5. FT-IR spectra of curcumin-loaded electrospun fibers based on gelatin and zein, both in the presence and in the absence of GTE. The spectrum of the commercial GTE is also shown. Spectra have been offset for clarity.

The increase in the relative absorbance in the region from 1000 to 1300 cm^{-1} was observed in the electrospun coatings containing GTE, apart from the presence of the band at $\sim 1637 \text{ cm}^{-1}$,

corresponding to stretching vibrations of C-O in the benzopyran structure of polyphenols (Li, Sun, Luo, & He, 2015), thus evidencing the presence of this polyphenol-rich extract within the fibers. However, the bands attributed to the GTE which appeared in the range from 1300 cm^{-1} to 900 cm^{-1} shifted to higher wavenumbers in the protein fibers, especially in the gelatin coatings. Similar spectral changes were observed when GTE was microencapsulated within electrosprayed gelatin and zein capsules (Gómez-Mascaraque, Hernández-Rojas, et al., 2017), and were attributed to the presence of intermolecular interactions between the proteins and the components of the extract. Indeed, both gelatin and zein have been previously described to interact with polyphenol molecules through hydrogen bonding and hydrophobic interactions (Li et al., 2009; Peña, De La Caba, Eceiza, Ruseckaite, & Mondragon, 2010). These interactions between the GTE and the proteins were also evidenced in the displacement of some of the characteristic bands of the proteins when the extract was incorporated. Specifically, the Amide I band of the proteins shifted to higher wavenumbers (from 1629 to 1638 cm^{-1} in gelatin and from 1647 to 1653 cm^{-1} in zein).

3.2.2. Thermal properties

Figure 6 shows DTG curves of the prepared coatings. Although the incorporation of curcumin within the zein fibers did not modify their thermal stability, most probably due to the low curcumin to zein ratio employed, the degradation of the gelatin fibers occurred at a slightly lower temperature when they were loaded with curcumin. In this latter case, the bioactive compound was incorporated in the form of liposomal curcumin, and phospholipids have been previously described to interact with proteins through hydrophobic interactions, altering their secondary structure (Kasinos et al., 2013).

Figure 6. DTG curves of gelatin-based fiber mats (A), zein-based fiber mats (B) and the raw additives incorporated within the fibers (C).

When the GTE was incorporated, the thermal stability of the curcumin-loaded gelatin fibers slightly increased again, counteracting the destabilization impact of the liposomal curcumin, despite its lower temperature of maximum degradation rate (cf. Figure 6C). This suggests that the interactions between GTE and the protein (cf. Section 3.2.2) had a stabilization effect on

the gelatin fibers, which also had an impact on their release and stabilization properties (cf. Sections 3.4 and 3.5). On the contrary, the zein coatings started degrading at slightly lower temperatures when GTE was incorporated within them, which indicated that the addition of the polyphenol-rich extract to the formulation did not have a positive impact on the thermal stability of the zein-based fiber mats.

3.3. Stabilization of curcumin

The incorporation of curcumin within electrospun protein fibers was expected to effectively increase the stability of this sensitive bioactive compound, as previously reported for other protein-based encapsulation structures obtained through electrohydrodynamic processing (Gómez-Mascaraque, Casagrande Sipoli, et al., 2017). In order to study this stabilization effect, the developed curcumin-loaded coatings as well as the free curcumin were immersed in PBS (pH = 7.4), a medium in which curcumin has been described to be unstable undergoing rapid hydrolytic degradation (Chen et al., 2009). Figure 7 shows the decay experienced by the absorbance of curcumin at 427 nm due to its degradation.

As expected, the free curcumin quickly degraded in PBS, being its absorbance completely lost after only 72 h. In contrast, microencapsulated curcumin exhibited improved stability, as it showed minimal absorbance decay throughout the experiment.

The results also showed that, in the absence of GTE, both proteins exerted a similar protection effect on curcumin during the first 24 h, but zein exhibited enhanced protection at longer exposure periods. Although curcumin in the gelatin fibers was incorporated within liposomes and, hence, an additional protective barrier was expected to be provided by the liposomal bilayers (Frenzel, Krolak, Wagner, & Steffen-Heins, 2015; Gómez-Mascaraque, Casagrande Sipoli, et al., 2017), gelatin is a more hydrophilic protein than zein, being susceptible to greater swelling degrees and therefore accelerated release of the bioactive

compound (cf. Section 3.5). According to the results, the higher curcumin release rate from the gelatin fibers could only be counteracted by the additional protection exerted by the liposomes during the first day of treatment. At longer exposure times, the limited solubility of zein provided enhanced protection to curcumin.

When GTE was incorporated within the gelatin fibers, an improved curcumin protection was observed. This was attributed to the intermolecular interactions established between the polyphenol rich-extract and the protein, as inferred from the infrared spectroscopy analysis (cf. Section 3.2.2), which resulted in an improved stability of the gelatin fibers (cf. Section 3.2.3). Since polyphenols have been proposed as natural crosslinkers for proteins (Anvari et al., 2016), it was hypothesized that the observed stabilization effect of the GTE on the gelatin fibers would also contribute to a delay in the release of curcumin from the gelatin coatings (confirmed in Section 3.5), and, thus, to an enhanced protection.

On the contrary, when GTE was incorporated within the zein fibers, the protective effect of these fibers was deteriorated, as greater curcumin degradation was observed. Although zein could also establish certain intermolecular interactions with the extract (cf. Section 3.2.2), these did not seem to be strong enough to improve the thermal stability of the zein coatings (cf. Section 3.2.3) which, in fact, slightly decreased. Similarly, the protection exerted by the zein fibers on curcumin was negatively affected by the addition of GTE. This could be explained in the light of the high hydrophilicity of the water-soluble extract, which could have increased the affinity of the materials towards aqueous solvents, facilitating the release of curcumin molecules (cf. Section 3.5). It is also possible that the interactions between zein and GTE (demonstrated through the FTIR results) competed with the interactions between zein and curcumin, thus partially precluding the stabilization effect of the prolamin over the bioactive compound.

Overall, the gelatin coatings containing GTE showed the greatest curcumin stabilization effect during the first 48 h of exposure to PBS, due to the contribution of both the crosslinking effect of the polyphenol-rich extract on the protein and the additional protection exerted by the liposomes. However, after longer treatment periods the gelatin-liposome combined structures started to lose their protective ability, and the zein fibers presented better protection, presumably due to their insolubility in aqueous solutions. Nevertheless, all the developed coatings demonstrated the ability to stabilize curcumin in slightly alkaline aqueous environments.

Figure 7. Degradation profile of free curcumin in PBS (pH = 7.4) as compared to that of curcumin incorporated within the developed coatings.

3.4. Curcumin release from the electrospun coatings

The ultimate goal of the developed coatings was to gradually release curcumin from the active packaging material to the actual food product to be preserved. Therefore, the diffusion of the bioactive compound from the protein fibers to two different food simulants was

assessed. 50% ethanol and a soy bean oil were selected as simulants for oil-in-water type foods (e.g. cream) and fatty foods (e.g. butter), respectively (10/2011/EC). Figure 8 shows the release profiles obtained for the different formulations in both food simulants.

Figure 8. Curcumin release profiles from the electrospun coatings in: (A) ethanol 50% (v/v); (B) soy bean oil.

In general, the gelatin coatings were able to release higher amounts of curcumin than the zein fibers and at a higher rate, despite the more hydrophilic nature of gelatin and the hydrophobic nature of the food simulants, probably facilitated by the presence of liposomal structures within the gelatin fibers. Proteins have been described to bind or interact with curcumin mainly through their hydrophobic residues (Araiza-Calahorra et al., 2018; Tapal & Tiku,

2012). Since curcumin was directly dissolved in the ethanolic zein solutions, it was readily available for interaction with this protein in the feed formulations.

Results showed that curcumin-zein binding delayed and prevented to some extent the release of curcumin from the zein coatings. In contrast, curcumin entrapment within liposomes in the gelatin formulations might have limited its direct interaction with this protein in the aqueous dispersions. As a result, curcumin exhibited a burst release from the gelatin coatings in 50% ethanol, aided by the swelling of gelatin in this medium and the good solubility of phosphatidylcholine in ethanol, achieving almost complete release after only 4 h of contact with the food simulant. Conversely, the release in this medium was more sustained from the zein coatings. Although an initial burst release was also observed in this case, this was followed by a more sustained curcumin release for up to 5 days, which is more desirable for the intended application.

Regarding the release in the soy bean oil, a more hydrophobic food simulant, it was considerably lower and slower for both proteins, since their swelling was much more limited in this oily medium. In this case, the curcumin release from the zein coatings was almost negligible, while gelatin fibers released up to about 18% of their curcumin content in a sustained manner.

Although the addition of GTE to the protein formulations had no significant impact on the curcumin encapsulation efficiency of the systems, it did have a considerable effect on the release properties of the coatings. As expected, the presence of GTE within the gelatin fibers decreased the release of curcumin in both media, especially in the soy bean oil, in which the release was almost negligible over the first month. This was attributed to the stabilization of the fibers due to the intermolecular interactions established between the polyphenols of the extract and the protein, as previously discussed (cf. Sections 3.2.2, 3.2.3 and 3.4). On the contrary, the release of curcumin from the zein coatings increased in 50% ethanol when GTE

was incorporated, although no significant effect was observed in the soy bean oil, due to the negligible release of curcumin from the zein fibers in this medium. These results are consistent with the reduced curcumin protection exerted by zein in the presence of the extract (Section 3.4), and were attributed to the increased affinity of the zein mats for the aqueous/alcoholic medium due to the hydrophilic character of the extract.

Overall, while the release in the more polar simulant was more sustained from the zein mats, gelatin would be a more suitable matrix for coating food packaging intended for the preservation of fatty food products. Therefore, each of the proposed systems is expected to perform better for a different type of food product, emphasizing the need of designing the food packaging coating specifically for the target food product.

3.5. Fiber stability assessment

The stability of the electrospun fiber coatings was assessed after immersion in water, a solvent in which both proteins are known to considerably swell. The changes in their surface morphology, as evaluated through SEM at different immersion time periods, are shown in Figure 9.

Fig. 9 SEM micrographs of electrospun coatings after immersion in water for different time periods.

Due to the high hydrophilicity of gelatin, this protein considerably swells (and eventually dissolves) in water. As a result, the fibrillar morphology of the electrospun gelatin coatings was completely lost after 60 min of immersion in water (Fig. 9). This is consistent with the fast release of curcumin from the gelatin mats in the most polar food simulant (cf. Section 3.5). Although the addition of GTE to the gelatin formulation effectively slowed down the

curcumin release in both assayed food simulants, it was not enough to prevent the gelatin fibers from collapsing in water. These results confirmed that, while the developed gelatin coatings showed a promising release behavior in contact with fatty food simulants, they were not adequate vehicles to achieve a sustained migration of curcumin to food products with high water content.

On the contrary, the stability of the zein fibers when immersed in water was considerably better as compared to gelatin. Although this protein is not soluble in water, electrospun zein fibers swell and collapse in water, as previously observed (Li et al., 2009).

Nevertheless, the fibrillar structure of the zein fiber coatings was maintained after 3h of immersion in water, although their morphology was altered to some extent (Fig. 9). This higher stability of the zein fibers in water is consistent with their better release performance in the polar food simulant, confirming that zein is a more adequate material for the design of bioactive coatings for packaging intended for food products with high water content.

4. Conclusions

In this work, curcumin-loaded nanofiber mats, with encapsulation efficiencies close to 90%, were produced from gelatin and zein solutions through the electrospinning technique. Due to the poor solubility of curcumin in aqueous solutions, a strategy based on its incorporation through liposomes had to be developed to generate the gelatin-curcumin fibers.

While free curcumin quickly degraded in PBS, microencapsulated curcumin exhibited improved stability. In particular, zein-based coatings exhibited enhanced protection at exposure periods longer than 24h and showed a more limited and slower curcumin release in hydrophobic food simulants. SAXS results evidenced that the liposome structure was preserved even after the solutions were transformed into dry fibers and that interactions were established between the gelatin and the surface of the liposomes. These interactions were

seen to be detrimental for the thermal stability of the gelatin fibers and responsible for the greater and faster release of curcumin in these coatings.

GTE-containing electrospun fibers were also produced in order to assess the impact of co-encapsulating a polyphenol-rich extract on the stability of the curcumin-loaded fibers, their protective ability and their release properties. The interactions between the GTE and the proteins were evidenced in the displacement of some of the characteristic bands of the GTE and the proteins in the FT-IR spectra from the hybrid fibers. In the case of the gelatin-based fibers, these interactions had a positive effect, improving their thermal stability and protective effect and decreasing the release of curcumin in food simulants. However, the addition of GTE was not able to prevent the collapse of the gelatin fibers in water, evidencing the fact that this system would be more suitable for the development of coatings intended for the preservation of fatty food products. On the other hand, the incorporation of GTE into the zein structures did not produce any significant improvements. The higher stability of the zein fibers in water and its better release performance in the polar food simulant, suggest that zein-based coatings would be more adequate for surfaces in contact with food products possessing high water contents.

This work has demonstrated that it is possible to develop coating structures based on protein fibers and polyphenol-rich extracts, such as GTE for the protection and sustained release of curcumin. These coatings can be optimized for packaging structures in contact with more hydrophilic or hydrophobic food products by selecting the suitable protein matrix.

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