Electrohydrodynamic processing for the production of zein-based microstructures and nanostructures

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Abstract

Recently, plant derived proteins have increased in interest and use due to a combination of interesting properties and industry-wide trends to replace animal derived proteins. Electrohydrodynamic processing (EHDP), can be used to develop micro- and nanostructures of plant proteins in a facile manner and, thus, increase their opportunities in the food, pharmaceutical, and biomedical industries. One of the most currently studied and promising plant proteins is zein. This review covers the most studied strategies to produce electrospun and electrosprayed zein-based structures. The most relevant properties, such as size, morphology, and surface area, are discussed according to the potential areas of interest, for instance in the food, biomedical, and pharmaceutical industries. In addition, applications of other electrospun/electrosprayed plant derived proteins are also presented, confirming the increasing interest of different industries in alternative proteins which can help promote sustainability.

Keywords: corn protein; plant proteins; electrospinning; electrospraying; food applications

1. Introduction

Food security can be described as the ability for people to have at all times physical, social, and economic access to sufficient amounts of healthy, safe, and nutritious foods. In addition, food preferences and the dietary needs for an active and healthy lifestyle should also be accounted for [1,2]. However, food insecurity has risen worldwide in parte due to global population growth that, in turn, will result in the need for agricultural expansion and an increase in agricultural productivity, causing extra pressure on the existing natural resources[1–3]. In particular, it is estimated that agricultural production will have to increase by 70 % to meet the demands of an increasing population, with meat and dairy production estimated to increase ~170 % and 150 %, respectively[1,3].

Alternative proteins have been emerging and gaining interest over the past few years and can help sustainability efforts. Studies have shown that animal derived proteins (e.g., meat and milk) are very inefficient, with a conversion ratio of vegetable food to meat or dairy protein of 7:1 (i.e., 7 kg of vegetable food are needed to produce 1 kg of meat or milk) [1,3]. Therefore, in addition to more sustainable production methods for animal derived protein, alternative sources of protein need to be considered to achieve a better balance between animal and other alternative proteins [3]. Changes to further emphasize the consumption of plant-based proteins can eventually lead to a better and more efficient distribution of high quality proteins for the world population, thus contributing to a future with improved food security [1–3]. Nevertheless, it will also be important to explore new processing technologies that could help overcome some technological changes when using plant-based proteins, thus increasing their applicability and consumption [4].

Alternative proteins can be obtained from vegetables (e.g., potato), cereals (e.g., corn, and wheat), seeds (e.g., quinoa, amaranth, sunflower, and chia), leaves (e.g., lucerne and moringa), legumes (e.g., peas, beans, soy, peanuts, and lentils), microalgae (e.g., *Spirulina*), fungi, and insects [1,3]. These proteins can have further use than those directly linked to food consumption and help alleviate stresses placed on the use of animal-derived proteins. Some of those uses are in biomedical applications since proteins derived from plants are inexpensive, readily available, biodegradable and can exhibit a lower immunogenic response than animal-derived proteins [5]. Some of these proteins are also biocompatible, which can favor applications in which cell seeding adhesion, migration, and proliferation are required. Proteins present several advantages when it comes to nanoencapsulation as they possess important functional and nutritional properties that other polymers might not, namely the wide range of surface functional groups that allow proteins to interact with a diverse group of substances, allowing for the production of micro- and nanostructures that can encapsulate hydrophilic and hydrophobic food bioactives [6].

Nevertheless, for alternative proteins to be easily used in food and biomedical industries, some issues need first to be addressed. For instance, in some applications, the low solubility and viscosity can represent a drawback (e.g., cultured meat and other structured foods) while, for others, structural modifications are required for proper use (e.g., development of micro- and nanocarriers, extracellular matrices, among others) [3,5,7]. As such, electrohydrodynamic processing (EHDP) arises as an option to overcome some of these drawbacks, as it allows to easily and cost-effectively produce micro- and nanostructures.

EHDP is a versatile top-down method that can be operated in two basic methods: electrospinning, which leads to the production of micro- and nanofibers, and electrospraying that, in turn, leads to the production of micro- and nanoparticles [8]. It has additional advantages when compared to other encapsulation methods such as high encapsulation efficiency, low cost, usage of room or ambient working conditions, as well as controllable temperature and humidity, if needed [8,9]. The basic setup for these two methods is the same and requires four different components. A syringe pump to force the controllable flow rate, a typically metal spinneret that usually consists of needles of varying diameters, a high voltage power supply that applies high fields to the tip of the spinneret, and a grounded metal collector where the samples are deposited [8,9]. Figure 1 shows a schematic illustration of the basic setup of EHDP (Figure 1a) and its different modes (Figure 1b).

The application of a voltage at the tip of the spinneret creates an electric field between it and the grounded collector. The applied electric field to the polymer solution, which is being forced through the metal spinneret, creates the so-called Taylor cone from which the polymer is stretched and twisted while the solvent quickly evaporates. At the end of the process, micro- and nanostructures are finally deposited on the grounded collector [8]. The development of the Taylor cone is essential for EHDP; however, it only occurs in a limited set of operational conditions. These conditions, or parameters, that influence EHDP can be categorized as solution, process, and ambient parameters, with the first two being the most important and more easily controllable ones [8]. Solution parameters include solvent type or mixture of solvents, polymer molecular weight (M_W) and concentration, solution viscosity, conductivity, and surface tension, whereas process parameters refer to three main variables, namely tip-to-collector distance, feed flow rate, and applied voltage [8,9]. Polymer concentration and M_W greatly influence the solution viscosity and are paramount for optimizing EHDP as noted by Silva et al. [9], where polymer solution viscosity was analyzed as a function of hydroxypropyl methylcellulose (HPMC) concentration and M_W. The resultant data were used to determine electrospraying and electrospinning zones, confirming that low-M_W polymers are more appropriate to produce particles, while high-M_W polymer tend to produce fibers.

Solvent choice can greatly influence properties such as viscosity, surface tension, and conductivity, all of them essential to process polymers by EHDP. Appropriate solvents should display good polymer solubility, adequate volatility that allows a proper solvent evaporation during the polymer flight from the tip of the spinneret to the collector, as well as having sufficient but relatively low surface tension [10]. In particular, having a low surface tension is a critical parameter for Taylor cone's formation during EHDP since the applied voltage must be able to overcome it [8–10]. Low surface tensions are, therefore, ideal and more adequate for EHDP, though a minimal or threshold value is needed to stabilize the process. It can be artificially lowered by adding surfactants or the mixture of different solvents, for example aqueous ethanol displays a lower surface tension than neat water [9,10]. Conductivity is another important parameter that can influence the outcome of EHDP, as extreme conductivities, either low or high, can hinder the EHDP or cause morphological changes in the produced micro- and nanostructures [10,11]. In regard to the process parameters, applied voltage and tip-to-collector distance are very intertwined since the electrical field in which the micro- and nanostructures that are produced can vary according to both parameters. For instance, increasing distance is usually accompanied by a need to increase voltage, whereas the use of inadequate distances might lead to partial solvent evaporation [11]. High voltages are habitually desirable as they can lead to the production of micro- and nanostructures with smaller diameters, however the use of excessive voltage fields might lead to a destabilization of the Taylor cone, resulting in undesired morphologies [11]. Solution flow rate is another important parameter, especially due to the fact that it is linked with process productivity. Typically, low flow rates are very common in electrospraying processes as they produce more spherical particles, while higher flow rates can lead to the production of beaded fibers and other undesired morphologies such as the production of micro-droplets [11]. Ideally, a flow rate that induces a steady-state, in which the feed flow rate equals the flow rate that is ejected from the spinneret tip, should be used [11]. Regarding the encapsulation or loading of bioactive compounds, EDHP is a very straightforward process. The polymer solutions are mixed with the bioactives that are intended to be loaded or encapsulated and are processed together. During the flight to the collector the solvent evaporates and the produced structures are deposited in the collector with the loaded bioactives [8–10]. When a co-axial spinneret is used, the selected bioactives (one or more) can be mixed with the inner flow, the outer flow, or both, allowing a greater control in the mixing of bioactives, polymers and solvents as well as higher protection for the bioactives [8,10].

As previously mentioned, a Taylor cone is seen in limited circumstances and conditions of operation. Thus, the resulting spraying or spinning cones can develop into different modes, namely with the effect of increasing voltage (when considering a fixed flow rate), which are summarized in Figure 1b). A categorization of these modes was described by Cloupeau and Prunet-Foch in the 1990’s [12]. When a low voltage is applied to the spinneret, dripping or micro-dipping (I) occurs, leading to the formation of large and fine droplets deposited on the collector, usually with low solvent evaporation. As voltage is increased, a spindle mode (II) can be observed in which, instead of normal droplets, elongated droplets (spindles) are ejected from the cone jet. With the appropriate voltage, a steady cone-jet mode (III) is obtained, leading to the formation of sprayed particles or spun fibers at micro- and nanoscale. This cone-jet mode can shift laterally, leading to an oscillating jet mode (IV) due to whipping instability as a result of a voltage increase. This oscillating jet mode can also turn into a multi-jet mode (V) when the increasing voltage splits the jet into multiple jets. In this regard, the number of jets tends to increase with the voltage [12].

Although EHDP is versatile, adaptable, and facile to use, there is no one generic or common EHDP setup or apparatus that is ideal for all types of polymers or desired morphologies. Therefore, this process needs to be studied and optimized according to the polymer used and intended applications. This review focuses on the need, design, and production of ultrathin systems materials based on zein and other alternative plant proteins using EHDP, that is, electrospun fibers and electrosprayed capsules. Their production, predominant solution and process parameters (e.g., flow rate, polymer concentration, voltage, tip-to-collector distance, etc.), and final properties of the resultant materials (e.g., release profiles, functional properties, cytotoxicity, etc.) are discussed. Additionally, an insight into the applications of the developed systems is presented, exploring their main advantages and drawbacks in industrial applications, namely in the food and biomedical industries, in the context of providing options for using alternative plant proteins.

2. Electrohydrodynamic processing of zein

Zein is considered the main storage protein in maize or corn, accounting for 35–60 % of total proteins and is found in the endosperm exclusively [13]. It constitutes 44–79 % of all endosperm proteins, depending on the variety of corn. However, technically zein is not a single polypeptide, but rather it represents a mixture of several proteins or polypeptides of various M_Ws that mainly vary in their solubilities. Therefore, according to their solubility, zein fractions can be classified as α (22 and 19 kDa), β (14 kDa), γ (27 and 16 kDa), and δ (10 kDa) [14]. Among these fractions, α-zein is the main one that, depending upon the genotype, accounts in corn for 85–75 % of the whole zein, whereas β- and γ-zein fractions only make up 15–10 % and 10–5 %, respectively [14]. This storage protein is considered a prolamin due to its high content of hydrophobic amino acids such as proline and glutamine and is soluble in aqueous ethanol, which is a sustainable solvent for electrospinning. Indeed, α-zein, which is the most commercially available, is insoluble in water unless specifically defined conditions are applied, which include the addition of alcohol, extreme alkali condition ($pH > 11$), high concentration of urea, and/or anionic detergents [15]. Among the four fractions, α-zein also shows the highest solubility in aqueous ethanol at contents of 50–95 % (w/w). In particular, a solution of 70 % (w/w) ethanol is the most optimal solvent used for zein extraction from corn, which is performed by means of a high temperature (60 °C) followed by chill separation [13]. A recent review presents the main characteristics of zein and its different conformation and characteristics according to the solvent and solubilization process used [16]. All the presented works used commercial zein with α-zein as the main fraction; however, it is important to mention that, according to the source, extraction, and purification process, different fractions balances can be obtained [17] and, thus, a different behavior during the EHDP could be observed.

Zein also presents the advantages of being renewable and biodegradable. However, this protein mixture is deficient in some amino acids, showing a negative dietary nitrogen balance and, more importantly, an absence of lysine and tryptophan. The imbalanced amino acid profile of zein results in poor nutritional quality that combined with its low water solubility represents the major obstacle for its direct application in food consumption [15]. Nevertheless, these drawbacks derived from the particular physicochemical properties of zein have also led to novel applications in a wide range of industries, especially in food and pharmaceuticals [13]. In the food packaging industry, zein has been extensively explored as a biopolymer to replace petrochemical polymers and it has been used as an edible coating material [13]. Furthermore, for pharmaceutical applications, zein is very promising for the development of water-resistant delivery vehicles due to its hydrophobic and unique solubility properties as well as its Generally Recognized as Safe (GRAS) status. Furthermore, zein has slower digestibility than other proteins and can form complexes and/or conjugates with other compounds [13]. These features certainly open up new opportunities in the production of micro- and nanostructures based on zein, such as capsules for drug and nutrient delivery with high potential for oral administration. In general, the selected preparation techniques to develop zein particles are based on one of the following procedures: liquid–liquid dispersion, solvent evaporation, or the antisolvent method. In the latter method, aqueous alcohol is first used to dissolve zein and, then, the resultant solution is poured or sheared into a water-based medium to cause phase separation and subsequent zein particle formation [18]. Another approach, which becomes particularly useful to fabricate zein nanoparticle-containing films at basic pH conditions, is solvent evaporation by means of the technique of cast drying [19]. Moreover, some industrial, scalable approaches for encapsulation and delivery applications have been recently reported to develop zein micro- and nanoparticles, including, for instance, the use of the supercritical antisolvent technique [19]. The latter method employs supercritical CO_2 ($ScCO_2$) to precipitate zein in the form of particles. However, unless a high flow rate of $ScCO_2$ is used as well as it is combined with 100 % methanol as the solvent, zein nanoparticles with uniform sizes are difficult to attain [19]. Therefore, this technology increases both the possible toxicity due to the presence of the organic solvent residue and the production cost. Furthermore, Li et al. [20] have also reported flash nanoprecipitation as a novel technology to prepare zein nanoparticles. This methodology uses solvent-antisolvent rapid mixing to fabricate colloidal nanoparticles. Nevertheless, some of these experimental setups have been so far limited to a laboratory scale, which can only process a small volume of zein.

In this context, the EHDP technologies (electrospraying and electrospinning) are relatively new and very promising for the production of capsules and fibers, respectively, at the micro- and nanoscale. However, EHDP also has its own limitations since the process parameters need to be optimized and, thus, the output, size, and morphology of the ultrathin zein materials can be greatly affected. In the next sections, the main solution properties and processing parameters for the electrospinning/electrospraying of zein are discussed.

2.1 Solvents

Aqueous ethanol is the main solvent used for processing zein by EHDP but other solvents have also been explored such as dimethyl formamide (DMF), methanol, isopropanol, acetone, and acetic acid [21,22]. Usually, the fibers produced from zein solutions using aqueous ethanol as solvent display ribbon-like morphologies, which is seen as a type of flat ribbons or ribbons with two tubes (dumbbell shape) and differs from classical fibers having a circular cross-section with a smooth surface. For instance, Chen et al. [21] originally produced ribbon-like bead-free zein nanofibers by electrospinning a solution of 30–50 % (w/v) zein in 70 % (w/w) ethanol. Results showed that with an increase in polymer concentration, zein fiber diameters increased from 1 to 6 μm. The formation of a ribbon-like morphology has been ascribed to an effect of ethanol fast evaporation, which first developed a layer around fibers that later was collapsed by evaporation of the remaining solvent. Some authors found similar suitable conditions leading to fiber-based morphologies without bead defects for 30–40 % (w/w) contents of zein in 80–90 % (w/w) ethanol. Moreover, it has been found that the resultant fiber size is nearly not affected by the amount of water present in ethanol as long as the prolamin is soluble in the solvent mixture, that is, within the range of 60–90 % (w/w) ethanol [23]. However, for a zein solution at 33 % (w/w), when the ethanol content increased from 50 to 96 % (w/w), outside the optimal range, it resulted in the increase of fiber diameter from 150 to 300 nm [24].

Similar findings were previously reported by Selling et al. [22], who also fabricated fibers by electrospinning from solutions of zein in 60–90 % (w/w) ethanol, leading to ribbon-like fibers with diameters between 1 and 8 µm. In particular, zein fibers with similar morphologies were attained during the electrospinning in 80 % methanol or 60–80 % aqueous isopropyl alcohol of 27–30 % zein. These results agree with the recent work of Moomand et al [25], who also explored aqueous isopropanol, showing a similar performance as that of ethanol for the electrospinnability of zein. Similarly, 27–30 % zein solutions in glacial acetic acid also resulted in ribbon-like fibers with diameters in the 1–5.6 µm range. Nevertheless, zein beads were found to occur instead in 60 % acetone or 60–90 % acetic acid when using 27 % zein. Furthermore, the authors also tested 40–27, 20, and 10 % (w/w) zein contents in DMF, 8-M urea, and 10 % NaOH, respectively, which did not succeed to provide electrospinnability [22]. In contrast to these results, zein nanofibers free of bead defects were formerly obtained from DMF solutions at 55–60 % (w/v) by Jiang et al. [26]. The authors also observed that electrospinnability improved as the concentration of zein was further increased.

It is also worth noting that combining acetic acid and ethanol resulted in flat fibers of zein [24]. This particular solvent mixture of ethanol/acetic acid 75:25 % (w/w) yielded electrospun platelet-like fibers with a mean cross-section of approximately 450 nm. Recently, the use of deep eutectic solvents (DES) has also been proposed for the electrospinning of zein as replacers of organic solvents such as DMF. For instance, zein nanofibers were electrospun from an optimal formulation of 45 % (w/w) in a choline chloride and furfuryl alcohol mixture with a molar ratio of 2:1 [27]. It was reported that, in contrast to hydrophobic zein nanofibers classically prepared in aqueous ethanol, zein nanofibers that were electrospun using DES displayed a super hydrophilic surface behavior with a finer average diameter, around 200 nm less.

2.2 Solution Properties

Some previous studies have indicated that polymer concentration is the most significant factor influencing fiber size and morphology. For instance, the effect of zein concentration and, hence, viscosity on the resultant electrospun fiber size and morphology was investigated by Neo et al. [28]. Zein solutions at 15 and 20 % (w/w) yielded viscosities of 22.1 and 64.2 mPa.s, respectively, which resulted in microbeads. However, from 25 % (w/w) zein solution (solution viscosity > ~100 mPa·s), bead-free and uniform fibers were attained. These results were ascribed to the fact that increasing viscosity promoted molecular entanglements and, thus, facilitated the formation of bead-free fibers. However, fiber diameter also increased for higher zein concentration, following a power-law relationship with a 3.6 exponent for 35–20 % (w/w) solution concentrations. In particular, during electrospinning at an applied voltage of 14 kV, a flow rate of 0.30 mL/h, and 10 cm of tip-to-collector distance, thick fibers with average diameters ranging from 910 to 628 nm were obtained from zein solutions at 35 and 30 % (w/w), respectively.

Similarly, in the study of Miyoshi et al. [29], zein solutions in 80 % (w/w) ethanol were processed by EHDP under 15 kV for concentrations in the 18–25 % (w/w) range. The authors observed a morphology composed of wrinkled beads with nanofibers bridging the beads at zein contents of 18 and 19 % (w/w). As similar to other studies, when zein concentration increased to 20 % (w/w) in the solution, fibers became thicker while the number of wrinkled beads was also reduced. Finally, ribbon-like fibers with a mean diameter of about 1 μm were formed when the zein concentration reached 21 % (w/w). Indeed, solution concentration was found to be the most significant factor controlling the electrospinnability of zein in a solution of 70 % (v/v) ethanol [21]. In particular, fiber diameter remarkably increased from 500 nm to 6 μm when zein concentration increased from 20 to 50 % (w/w). In the same way, it was found that protein concentration plays a major role in the fiber size of zein processed by electrospinning [24]. In this former study, 80 % (w/w) ethanol solutions with a zein concentration ranging from 5 to 50 % (w/w) were processed by EHDP keeping all the other process variables constant, that is, 11 kV, 0.37 mL/h, and 10 cm. It was observed that, in the zein concentration 5–12 % (w/w) range, electrosprayed zein nanobeads with sizes varying from approximately 100 to 220 nm were attained. Then, fiber formation was successfully produced from 25 % (w/w) and thickness changes were relatively low up to 40 % (w/w), resulting in fibers with an average diameter of approximately 200 nm. At contents of protein higher than 40 % (w/w), zein fiber diameter exponentially increased to values above 1 μm. It is also worth mentioning that, at low protein contents, a flat ribbon-like morphology was developed by the fibers, whereas they changed to a tubular-type morphology with some split nanofibers at high concentrations. More recently, zein nanofibers were obtained by needle-less electrospinning with rotating spiked-like spinneret using a zein solution at 13 % (w/v) with a voltage of 21 kV [30]. It was observed that higher values of solution viscosity and electrical conductivity were obtained by increasing zein concentration, which resulted in the production of fibers with larger diameters.

Figure 2 shows, as a proof of concept, the effect of concentration on electrospinnability. SEM micrographs show different zein-based structures obtained from EHDP of solutions with zein concentrations of 12, 25, 33, and 42 % (w/w) in 80 % (w/w) ethanol. Lower (5 % (w/w)) and higher contents (50 % (w/w)) were also attempted, but micro dripping and blocking, respectively, impaired the process. In Figure 2, it is possible to differentiate the different morphologies that can be obtained in EDHP. It can be observed in the SEM images that at low concentration, that is, 12 % (w/w), nanobeads of zein with sizes ranging of 500–50 nm were formed due to the low solution viscosity (Figure 2a). When the concentration was increased to the intermediate value of 25 % (w/w), wrinkled microbeads and nanofibers bridging the beads were produced (beads-on-a-string or beaded fibers, Figure 2b). Thereafter, higher concentrations led to the formation of ribbon-like bead-free fibers (Figure 2c), which were optimal in terms of size diameter (mean diameter <1 μm) at 33 % (w/w). However, at 42 % (w/w), the size diameter of the zein fibers considerably increased, yielding to the formation of microfibers of 1–3 μm, with also a ribbon-like morphology (Figure 2d), based on the fact that the intrinsic viscosity of the protein solutions generated was too high.

2.3 Processing conditions

During electrospinning, the high voltage creates an electrically charged jet of polymer fluid that is ejected from the tip when the electric field reaches a critical value. In this regard, Miyoshi et al. [29] firstly indicated that the critical voltage of zein solutions at 25 % (w/w) in 80 % (w/w) ethanol is about 8 kV. However, when the concentration of zein was reduced below 21 % (w/w), higher voltages of 30 kV were needed to obtain bead-free fibers.

In a previous research study, the process parameters, that is, voltage, flow rate, and tip-to-collector distance, were also analyzed as a function of the zein fiber size, considering the thinnest, average, and thickest diameters [24]. Figure 3 gathers the evolution of the fiber diameter versus the power voltage (Figure 3a), flow rate (Figure 3b), and distance between the tip and collector (Figure 3c), for the electrospinning of a zein solution at 33 % (w/w) in 80 % (w/w) ethanol at the stable environmental conditions of 24 °C and 60 % RH. In terms of voltage, one can observe that fiber diameter nearly doubled in size when this parameter was increased from 7.5 to 12.5 kV, using a constant flow rate of 0.37 mL/h and a tip-to-collector distance of 10 cm. However, higher voltages led to a slight increase in the fiber size. This observation is due to the fact that an increase in the volumetric flow rate is produced when the applied voltage goes beyond a certain level. A similar phenomenon was described by Miri et al. [31], who ascribed the effect of voltage to a reduction in the flight time of the electrospun jet during zein fiber production by electrospinning.

In relation to the flow rate, it was observed that there is a low influence on the fiber diameter up to values of 0.45 mL/h. For flow rate values of up to 0.45 mL/h, the mean fiber diameter was kept nearly constant at 200 nm when voltage and a tip-to-collector distance were fixed at 11 kV and 10 cm, respectively. Similar results were reported by Miri et al. [31], where the variation of the flow rate in the 4-12 mL/h range of a zein solution at 26 % (w/v) did not significantly affect the fiber diameter using a tip-to-collector distance of 15 cm. Nevertheless, the fiber average diameter increased largely, up to approximately 700 nm, when the volumetric flow rate was increased to 0.5 mL/h. This effect was ascribed to a threshold volume charge density value from which the fibers merge in flight. Finally, the zein fiber morphology was not altered notably with varying the distance between the tip and collector in the range of 5-13 cm for 11 kV and 0.37 mL/h. In contrast to the other process variables, beyond a threshold value of around 13 cm, the zein fiber diameter decreased from approximately 250 to 150 nm. This finding was related to a combined effect of an electric field strength decrease with the increase of the solvent evaporation rate.

3 Applications of electrospun/electrosprayed zein-based micro- and nanostructures

As shown above, EHDP is a versatile technology that can produce different micro- and nanostructures based on zein. Since corn zein has GRAS status, its applications in food, pharmaceutical, and biomedical areas have risen in the last years. The main applications of electrosprayed/electrospun zein materials are related to encapsulation or immobilization of an active compound, but there are also other applications where the stand-alone zein-based structure can be used [32]. In the next sections, some of the most explored applications of electrospun and electrosprayed zein structures are presented.

3.1 Food applications

In the food industry, the use of EHDP of zein has been focused on two main applications: fiber-based packaging material (electrospinning) and encapsulating active compounds (mainly electrospraying). For the latter application, electrosprayed zein nano- and microcapsules containing bioactives can be added to foods to functionalize or enrich them, envisioning their delivery in the human gut or to improve the preservation of foods [32]. In packaging applications, however, the main focus has been placed on electrospun fiber mats that work either as a barrier interlayer within a multilayer packaging structure to increase the performance of bioplastics or as an active coating incorporating antimicrobial or antioxidants compounds [33]. The latter concept was exemplified by Cerqueira et al. [34] by the development of multilayer systems in which cinnamaldehyde was incorporated into zein fibers and the antimicrobial capacity of the electrospun mats was tested against *Listeria monocytogenes*. Besides being used as a carrier for the active compound, the zein was also used as an interlayer for the formation of multilayer systems using different biopolymers, as illustrated in the SEM micrographs of Figure 4. Figure 4a shows the electrospun fibers on the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)-based film, whereas Figure 4b shows the multilayer system with sodium alginate-based film and PHBV films and zein as interlayer. For the formation of the multilayer, a hot press was used at 130 °C during 2 min.

In the context of food applications, Amjadi et al. [35] loaded rosemary essential oil in combination with zinc oxide nanoparticles (ZnO NPs) into hybrid zein/κ-carrageenan nanofibers and showed that this novel nanocomposite can be used as an active material against *Staphylococcus aureus* and *E. coli*. Also, Amjadi et al. [36] showed that zein, in combination with alginate, was able to form ultrathin fibers filled with titanium oxide (TiO_2)

and betanin, which can display antibacterial activity to food-borne pathogenic bacteria, *E. coli* and *S. aureus*, and also antioxidant activity. Despite the nanoscale and the active compounds used, the biocompatibility of fabricated nanofibers after *in vitro* cell cytotoxicity assay was also demonstrated.

In addition to several works that performed antibacterial and antioxidant tests *in vitro*, there were also some applications in real foods. For instance, Li et al. [37] developed gelatin/zein fiber mats encapsulated with resveratrol and used them for the preservation of pork. In another study, Shao et al. [38] showed the capacity of zein nanofiber mats loaded with cinnamaldehyde essential oil to extend the shelf life of *Agaricus bisporus* mushrooms. The authors compared this novel active electrospun mat with low-density polyethylene (LDPE) and non-packed mushrooms and observed that the newly developed active mats effectively prolonged food shelf life. Likewise, Niu et al. [39] produced hybrid fibers based on zein and ethylcellulose and loaded them with cinnamon essential oil. The study showed that these electrospun fiber mats can be potentially applied to reduce weight loss and maintained the firmness of the *Agaricus bisporus* mushrooms and, thus, improve their quality during 6 days of storage at 20 °C. Another interesting work was presented by Böhmer-Maas et al. [40], aiming to develop active packaging using electrospun zein nanofibers. They showed the possibility of using these nanofibers for the immobilization of TiO_2 as ethylene scavengers during cherry tomatoes storage.

The use of zein-based nanofibers for the encapsulation of indicators in intelligent packaging has also been tested. Interesting results were obtained for time-temperature indicators (TTI) using immobilized laccase [41] and also for the spoilage monitoring on different food products, such as trout fish using alizarin color [42]. Following applications in packaging, the encapsulation of bioactive compounds for further incorporation in food is one of the most explored applications of zein-based structures produced by EHDP. Some bioactive compounds with antioxidants properties were encapsulated, such as phenolic-enriched extracts from pulp, seed, and skin of orange chilto [43] and saffron extracts [44]. Alehosseini et al. [44] reported that both ultrathin fibers and particles produced by EHDP prevented ultraviolet (UV) degradation of the extracts when compared with non-encapsulated extracts. They also showed that the electrosprayed structures displayed a better protective behavior when compared to the electrospun structures, which was explained by the larger diameter of the particles and, thus, a greater shielding effect.

Zein hydrophobic behavior and solubility in ethanol also led several researchers to explore it for the encapsulation of bioactive lipophilic compounds that, in their free form, present low solubility in aqueous environments and low bioavailability in the human gut. Based on their partial digestibility in gastric conditions, zein-based nanoparticles successfully increased the bioavailability of encapsulated bioactive compounds of beta-carotene [18]. Additionally, Bushani et al. [45] showed that green tea catechins can be effectively encapsulated by electrospraying using zein as matrix. Using 5 % of zein solution (80 % ethanol), the authors obtained monodisperse nanoparticles with a mean diameter of 157 nm. The study also showed that the encapsulated green tea catechins were more stable under *in vitro* gastrointestinal stability and easier to permeate through the Caco-2 cell monolayer when compared with unencapsulated catechins.

One recent study also explored the possibility of using zein fibers as a fibrous structure, envisioning applications in whole-tissue meat analogs. They compared three methods (i.e., electrospinning, antisolvent precipitation and mechanical elongation of self-assembled zein networks) to produce these 3-dimensional structures and showed that each method has unique features that need to be considered for further developments. Despite the low throughput of the electrospun fiber they showed that zein fibers presented an increased hardness and chewiness when compared with zein in particulate form, that was explained by the zein fibers higher surface area [46].

3.2 Biomedical and pharmaceutical applications

The use of biocompatible materials, such as zein, can also be of great interest in the biomedical field. Therefore, several works have explored ultrathin zein fibers in this field as, for instance, drug delivery systems in wound healing and tissue regeneration of cartilage, bone, heart valves, muscle, neural tissue, and skin [47]. In this particular field, the mats composed of ultrathin fibers produced by electrospinning can potentially mimic the morphological characteristics of human tissues (e.g., extracellular matrix of the skin). Therefore, several electrospun zein-based microstructures have been tested for tissue regeneration. For example, Yang et al. [48] evaluated the capacity of zein/gelatin blends to produce nanofibers to be used as a scaffold to human periodontal ligament stem cells. It was proved that the presence of gelatin is important for the adhesion and spreading of human periodontal ligament stem cells, which was explained by the hydrophobicity decrease of the mats. Miao et al. [45] modified zein using poly(L-lysine) and produced nanofibers obtained thereof as a scaffold for neural stem cells. It was demonstrated that fibers produced with higher amounts of poly(L-lysine) are the best candidate in terms of viability, adhesion, proliferation, and differentiation of neural stem cells. Another potential application that takes advantage of the unique structures produced by the electrospinning of zein is wound healing. Furthermore, the use of electrospun zein-based fiber mats for cell growth and the combination of these 3D structures with active compounds have been tested in different configurations and blended with different biopolymers aiming to reduce microbiological growth. For instance, Ghorbani et al. [49] developed a scaffold based on zein, poly(ε-caprolactone) (PCL), and collagen loaded with ZnO NPs and *Aloe vera* that displayed antimicrobial properties, while Liu et al. [50] developed zein and poly(ethylene oxide) (PEO) membranes for application in wound healing, which were loaded with thyme essential oil that promoted the wound healing process. Furthermore, Liu et al. [51] developed a cellulose acetate-zein composite nanofiber membrane that, when loaded with sesamol, enhanced wound recovery in diabetic mice. Similarly, Rad et al. [52] prepared a PCL, zein, and gum Arabic scaffold through multilayer electrospinning loaded with *Calendula officinalis* extract, which displayed antibacterial properties. Table 1 offers more details of the research works that were briefly described herein, regarding applications developed in the last years in the wound healing field using electrospun zein as the base material.

In the pharmaceutical industry, one of the major challenges is the development of effective delivery systems for different types of drugs that present low water solubility as well as poor absorption. This is critical when these drugs are taken via oral administration. Therefore, encapsulation processes have been presented as a way to improve this dispersibility as well as doubling as a strategy to increase their bioavailability and absorption. In this particular case, zein micro- and nanostructures are of great interest due to their hydrophobicity, biodegradability, and biocompatibility. In this regard, Heydari-Majd et al. [53] encapsulated bioactive Barije essential oil in electrospun zein nanofibers. The resultant electrospun mats were proposed as novel delivery systems due to their enhanced anti-diabetic and antioxidant properties. Also, Lee et al. [54] developed a tri-layered nanofiber mat by electrospinning to control the release of ketoprofen drug. This novel study reported the use of a tri-layered nanofiber mat composed of zein, as the external layers, and poly(vinylpyrrolidone) (PVP) blended with graphene oxide (GO) as the middle layer, in which the drug was incorporated into all the layers. The authors showed that by controlling the thickness of the external layers, it was possible to control the release of ketoprofen. Finally, zein nanofibers have also been tested for the encapsulation of small interfering ribonucleic acid (siRNA) [55]. Results showed a loading efficiency of 58.57 % and higher stability of siRNA at room temperature for a longer period of time. Moreover, the prepared electrospun zein nanofibers supported the cell adhesion while facilitating the transfection of siRNA into the cells.

4 Electrohydrodynamic processing and applications of other plant-based proteins

For protein solutions to be electrospinnable, in addition to displaying high solubility, proteins should display a random-coil behavior instead of globulin-like behavior. Another requisite for the electrospinning of proteins is protein unfolding and, as such, the choice of solvent in which to prepare the protein solutions has to be carefully thought out as the solvent needs to both solvate the protein and induce its unfolding [56]. These requirements are rarely met by plant proteins, which usually have a globular native state that creates added difficulties due to high hydrophobic and ionic interactions, hydrogen bonds, a complex network structure, and low charge density. Additionally, upon denaturation, proteins with globular native state typically form insoluble aggregates [7,56]. As such, working with most plant proteins is a challenge and, to overcome these obstacles, combining these proteins with other easily spinnable polymers is a possibility. In addition to finding the appropriate solvent mixtures that allow for protein solubilization and unfolding, it has been suggested that the presence of a spinnable polymer might help in the electrospinning of plant proteins. The chains from spinnable polymer structures combined with the non-electrospinnable proteins form an intertwined network in which the non-electrospinnable proteins are contained, thus, creating a mixed system that can be electrospun [56]. Zein, despite being a globular protein, like other alternative plant proteins, is water insoluble. Even though it has some of the same issues regarding spinnability than other plant proteins have (e.g., being a globular protein), zein undergoes a partial unfolding process when dissolved in aqueous ethanol solutions, inhibiting protein aggregation, which results in zein being soluble in aqueous ethanol, and as such electrospinnable [16,57]. Recently, other plant proteins have been explored in EHDP. These plant proteins offer different characteristics and nutritional profiles than zein and include soy, pea, bean, amaranth, wheat, and microalgae protein [56,58–61]. Examples of the micro- and nanostructures produced by EDHP processing of these plant proteins can be seen in Figure 5.

In this context, Kutzli et al. [58] electrospun a biopolymer blend of maltodextrin and/or whey (WPI) and soy protein isolate (SPI) and assessed the influence of the protein type and ratio on the fiber morphology. SPI is mostly composed of two globular storage protein fractions, β-conglycinin (7S) and glycinin (11S), and it is of great interest to the food industry due to being inexpensive and of high nutritional value [58]. The authors reported that the use of SPI decreases the surface tension of the solutions used for electrospinning whereas it increased conductivity and viscosity when compared to the neat WPI solutions. The increase in viscosity of the SPI solutions led to the production of fibers with larger diameters than those of neat WPI fibers (4.74 µm and 2.85 µm, respectively), and with lower and uneven production rates (0.5 ± 0.25 g/h for SPI and 0.98 ± 0.13 g/h for WPI). Once the authors removed the insoluble fraction from the SPI solution, a lower solution viscosity was obtained (accompanied by a decrease in fiber diameter, to around 2.62 µm) and better spinning results were observed with a more uniform production rate of 0.5 ± 0.06 g/h. These results confirm that both the protein properties (e.g., aggregation state) and type can directly influence the outcome of the size, morphology, and production rate of their electrospun fibers.

In another work, Aguilar-Vázquez et al. [56] studied the electrospinnability of pea and common bean (respectively *Pisum sativum* and *Phaseolus vulgaris* L.) proteins and how solvent choice impacted on the rheological and conformational properties of the protein solutions. Pea proteins are mostly storage proteins, with globulins the most abundant (up to 66 %), followed by albumins. The major fractions that compose globulins are legumin (11S; ~320-380 kDa), vicilin (7S; ~150-180 kDa), and convicilin (7–8S; ~290 kDa). Pea protein displays functional properties such as gelling and foaming that, combined with its hypoallergenic properties, make it interesting for use in the food and biomedical industries [56]. Results showed that solvent choice did influence the conformation of the proteins and their solution. The selected solvents were able to partly denature the protein isolates, although not to the point of turning the solutions electrospinnable, as most solutions either produced beads or a combination of beads and fibers. Protein denaturation, solution viscosity, and solvent vapor pressure played an important role in the electrospinnability of the solutions. Bean protein isolate demonstrated better performance in EHDP due to the bean protein isolate yielding a fiber-like morphology when dissolved in hexafluoroisopropanol (HFIP). Bean protein presents mainly phaseolin, accounting for 30 to 50 % of the protein content of beans. Other relevant proteins that are part of its composition are lectins, proteases, trypsin, and Bowman-Birk inhibitors [56]. In the study performed by Soto et al. [59] amaranth protein and pullulan nanofibers, loaded with nisin, were developed and their antimicrobial properties were assessed in apple juice and fresh cheese. Amaranth protein is mostly composed of albumin (~65 %) and globulin (~17 %), which are its main storage proteins having as the most relevant globulins: 11S, P, and 7S. Amaranth albumins are rich in valine and lysine, while globulins are rich in valine, leucine, and prolamin, among others [62]. Electrospun fibers were produced with a mean diameter of 120 nm with an encapsulation efficiency higher than 90 %. The produced fibers were able to release 81.5 % (12h at pH 3.4) and 44 % (12h at pH 6.1) of nisin. The nisin-loaded fibers displayed complete bactericidal activity against *Salmonella* Typhimurium (after 48 h), *L. monocytogenes* (after 20 h), and *L. mesenteroides* (after 48 h) in apple juice and fresh cheese (142, 120, and 170 h, respectively). The amaranth and pullulan fibers show a great deal of potential to be used as a food additive, edible film, or packaging material to control post-processing contamination of food and beverage products.

As shown above, this area is still relatively unexplored due to the particular electrospinnability requirements for plant proteins. However, the potential of using plant proteins (other than zein) is high, especially when considering the prospects of combining different proteins and biopolymers to overcome some of the requirements. Moreover, the EHDP setup and conditions can also be modified to favor the processability of the plant proteins. For instance, Moreira et al. [60] developed ultrafine fibers with antioxidant properties from microalgae (*Spirulina*, mostly consisting of protein, around 65 to 80 % (w/w) using free surface electrospinning, a method of high-throughput production of nonwoven fibers that does not require the use of needles, opposite to traditional electrospinning methods. Electrospun fibers were particularly produced from a mixture of *Spirulina* sp. LEB 18 protein concentrate and PEO and loaded with 2 % (w/w) of phycocyanin. Fiber diameter was influenced by the protein content, showing diameters of 269, 314, and 542 nm for 5, 7.5, and 10 % (w/w) protein concentration, respectively, probably due to an increase in viscosity at higher protein concentrations. Fibers produced at higher protein concentrations were also less uniform in size. Phycocyanin-loaded fibers displayed higher antioxidant activity than the fibers without the pigment, with the active fibers displaying a 29.7 % reduction and 5.3 % inhibition of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) cation ($ABTS^+$) and 1,1-diphenyl-2-picryl hydrazyl (DPPH•) radicals, respectively. The developed phycocyanin-loaded fibers might be useful for food and biomedical industries for the protection of oxygen-sensitive bioactive compounds that doubles as an enriched source of plant protein. In another study, Mendes et al. [61] studied the effect of different solution properties on EHDP of potato protein for the development of both particles and fibers. Potato proteins are classified into three major protein categories, namely patatins (which account for close to 40 % of the soluble proteins), protease inhibitors (50 % of total soluble proteins), and high-M_W proteins (around 10 % of total soluble protein). Authors studied the effect of protein concentration and solvent selection, whereas water and aqueous ethanol and water-glycerol mixtures as well as HFIP were used as solvents. For particle production, all solvents were deemed appropriate, although different protein concentrations were needed according to solvent use (e.g., 5 % (w/v) for HFIP, but at least 20 % (w/v) was needed when using water as the solvent). Particle diameter ranged between 0.3 to 1.4 µm, with water being the solvent that produced particles with the lowest diameter, followed by the aqueous ethanol, and the water and glycerol mixture, whereas HFIP yielded the largest particles. Water-based solvents were unable to produce fibers, while only samples prepared in HFIP developed a fiber-like morphology. At 10 % (w/v) potato protein concentration, fibers displayed an average diameter of 0.17 µm, while at 20 % (w/v) % fibers displayed a flattened morphology with an average diameter of 1.72 µm. Vitamin B12 was also loaded into the fibers produced at 20 % (w/v) potato protein concentration (the average diameter increased to 2.01 µm) and the electrospun fibers were evaluated regarding their release properties in phosphate-buffered saline (PBS) at 37 °C. Potato protein fibers were found to be insoluble in aqueous PBS, which was attributed to protein unfolding during the electrospinning process. Results showed an initial burst release of 50 % of vitamin B12 content in the first hour, followed by a slow and sustained release up to reaching ~70 % after 17 h.

5 Future trends and final remarks

EHDP is fast becoming one of the most interesting processes to produce micro- and nanostructures for food and biomedical areas. The use of different plant proteins, such as zein, alone or in combination with other biopolymer or additives, and the development of multilayered systems raise up the possible applications since different structures with dissimilar morphologies can be developed. It has also been demonstrated that the solvent type, solution properties, and processing conditions can all together determine the processability of the protein and the final characteristics of the resultant materials. Therefore, from the above, the attained zein materials obtained by EHDP can vary according to the setup and conditions and, although some trends exist regarding the influence of some variables in the structures' characteristics (e.g., an increase in concentration requires an increase in voltage and results in micro- and nanostructures with larger diameters), a particular optimization for each process might still be needed. Nevertheless, since the optimization process can be relatively fast and easy to implement in most laboratories, this should not be a major issue. The use of electrospun/electrosprayed zein materials also shown to be increasing in different areas of application. Moreover, the uses of other plant proteins (e.g., soy, pea, bean, amaranth, wheat, potato, lentil, sunflower, and microalgae protein, among others) are also of recent interest for the food and biomedical industries and, as such, their research is of extreme importance.

However, several issues still need to be solved. Some of these concerns include, for instance, the homogeneity and throughput in large volumes of electrospun/electrosprayed zein materials, the availability of a constant and stable source of plant proteins, the release mechanisms of the resultant materials and systems as well as the detailed assessment of the digestion process and the safety of the resultant structures. The lack of electrospinnability of additional alternative plant proteins is also a concern, regarding their stand-alone use. For example, an ideal combination of solvents needs to be found (for each protein) in order to ensure protein solubilization and protein unfolding. This will allow improving their electrospinnability and their stand-alone use. As previously mentioned, an alternative to overcome the low electrospinnability of plant proteins is the combination of proteins with easily electrospinnable polymers, for instance PEO, poly(vinyl alcohol) (PVA), and pullulan, as demonstrated in some of the most recent research, creating a spinnable mixed system of protein and polymer. All these issues will have to be faced and further explored in order to make use of the real potential of plant protein-based micro- and nanostructures obtained by EHDP and, as such, more research into the processability of alternative plant proteins should be conducted over the next years to step towards increasing their use.

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Author contributions

P.M.S., S.T.-G., and M.A.C undertook literature search and manuscript drafting. P.M.S, S.T.-G, and M.A.C. wrote the manuscript. All authors discussed, reviewed, edited, and approved the manuscript. S.T.-G., A.A.V., and M.A.C. were responsible for funding.

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The authors analyzed the influence of protein type (animal or vegetable) as well as the protein ratio on the morphology of electrospun fibers. The use of soy protein isolate hindered the fiber production process, when compared to the whey protein isolate, but upon the removal of the insoluble fraction of the soy protein isolate the spinning process was improved. This work was able to correlate process spinnability and fiber morphology to the protein content in the solutions.

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The authors developed amaranth-nisin electrospun nanofibers that were assessed regarding their antimicrobial effect in apple juice and fresh cheese. Nisin release was dependent upon pH and was diffusion driven. The encapsulated nisin decreased 6 Log of microorganism populations in apple juice and completely inactivated them in Panela cheese. The developed nisin-amaranth-pullulan mats can thus be used for packaging materials for beverages and cheese.

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The authors developed electrospun ultrafine fibers from Spirulina sp. LEB 18 protein concentrate, through a novel free-surface electrospinning method that allows for high throughput production of fiber mats. The electrospun fibers were loaded with an bioactive pigment extracted from microalgae (phycocyanin) that displays antioxidant activity, and as such the developed nanofibers can potentially be used as a bioactive material.

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