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Nanostructuring Biopolymers for Improved Food Quality and Safety

Laura G. Gómez-Mascaraque*, Maria J. Fabra*, Jinneth L. Castro-Mayorga*, Gloria Sánchez*.,***, Marta Martínez-Sanz†, Amparo López-Rubio*

**Institute of Agrochemistry and Food Technology (IATA-CSIC), Valencia, Spain;*

***University of Valencia, Valencia, Spain; †Bragg Institute, Australian Nuclear Science and Technology Organisation (ANSTO), Kirrawee DC, NSW, Australia*

1 Biopolymer-Based Nano- and Microencapsulation Matrices for Bioactive Protection

The use of microencapsulation matrices for food applications is limited to edible, biodegradable, and preferably inexpensive materials, with biopolymers being the ideal candidates that meet these requirements. Both proteins and polysaccharides have been proposed as promising vehicles for the protection and/or delivery of bioactive ingredients, each of them with their own advantages and drawbacks.

1.1 Carbohydrates

Among carbohydrates, that is, monosaccharides, oligosaccharides, and polysaccharides, the latter are considered the most suitable as encapsulation matrices due to their large molecular structure, which allows effective entrapment of bioactive ingredients (Fathi et al., 2014). Polysaccharides are very abundant and relatively inexpensive biopolymers (Mohan et al., 2015). They also possess different reactive groups in their structure, which facilitate interaction with a range of bioactive compounds and even allow functionalization of their backbone to achieve tailored functionalities, including the possibility of designing self-assembling structures for encapsulation (Palao-Suay et al., 2016). It must be noted, however, that the reactivity of their functional groups has also been envisaged as a disadvantage for the encapsulation of certain bioactive ingredients capable of reacting with them, generating toxic products and/or precluding their subsequent release (Mohan et al., 2015). Polysaccharides are very versatile due to the variety of chemical structures, functional groups, and molecular weights available, and the presence of both hydrophilic and hydrophobic motifs along their

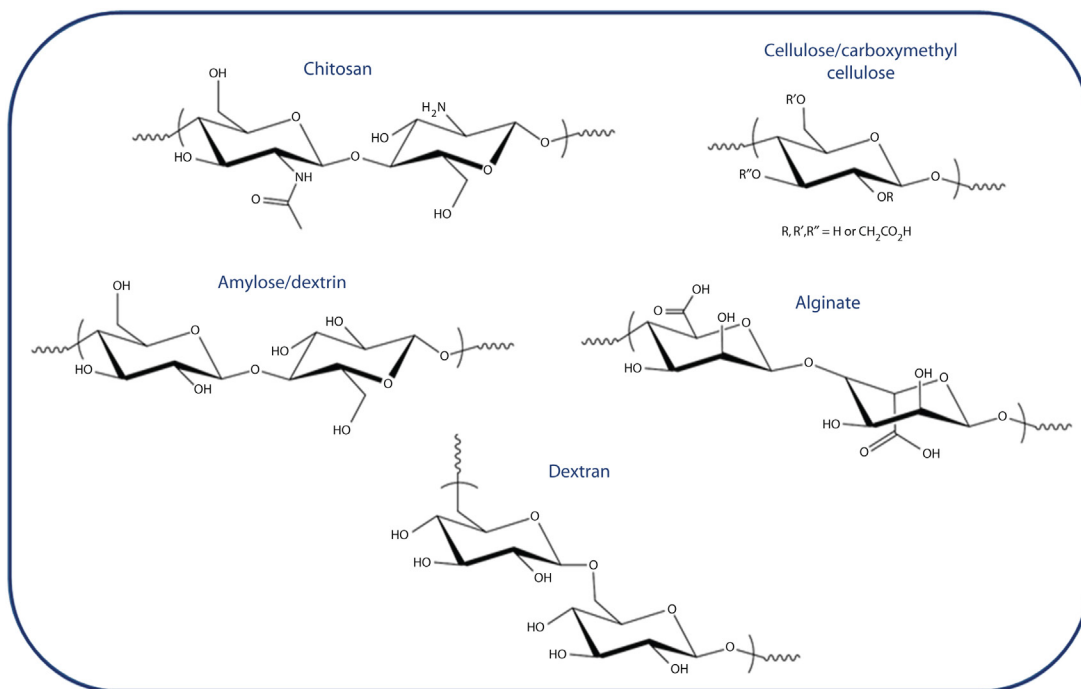


Figure 2.1: Chemical Structure of Some Polysaccharides Used for the Encapsulation of Bioactive Ingredients.

molecular chains (Santiago and Castro, 2016). Fig. 2.1 shows the chemical structure of several polysaccharides that have been widely explored as encapsulation matrices. Other commonly used carbohydrates, such as pectins or some gums are a heterogeneous group of polymers and thus its chemical structure has not been represented. Another interesting feature of polysaccharides is the great stability of their structure, even under harsh temperature conditions, in contrast with proteins, which can be denatured during material processing at high temperatures. Furthermore, a number of polysaccharides are considered dietary fibers, being resistant to enzymatic degradation during oral, gastric, and small intestine digestion but vulnerable to microbial fermentation in the colon (Fathi et al., 2014). Most of the commonly used polysaccharides for encapsulation have a plant origin, such as starch, pectin, cellulose, alginate, or guar gum, but animal-derived polysaccharides, such as chitosan or those from bacterial origin, such as dextran are also widespread for this application. An exhaustive review highlighting the advantages and disadvantages of each of them as delivery vehicles in food systems has been recently published (Fathi et al., 2014).

1.2 Proteins

Food proteins are excellent candidates as microencapsulation matrices because, in addition to the protective effects they might exert on the bioactive ingredients, they have a high

nutritional value and generally exhibit functional properties themselves (Ma et al., 2014), being considered the most nutritionally beneficial delivery systems (Mohan et al., 2015). Moreover, as Livney (2010) points out, some proteins are natural vehicles whose main function is to store, transport, and/or deliver essential compounds throughout the organism and, thus, they could also be exploited to deliver other molecules of interest. Especially taking into account that proteins are capable of binding both hydrophobic and hydrophilic small molecules (Considine and Flanagan, 2009), and they have self-assembling properties, surface activity, and good gelation characteristics (Livney, 2010). In general, proteins from animal origin, especially dairy proteins, but also gelatin and egg proteins, are more commonly employed than plant proteins for encapsulation purposes (Karaca et al., 2015). Some of the advantages of milk proteins over plant proteins include their higher solubility over a broader range of pH, their lower molecular weight, and their greater flexibility. On the other hand, plant proteins have a better acceptance from some groups of consumers and are generally less expensive (Karaca et al., 2015). The most widely used milk proteins are caseins and whey proteins, and their application as encapsulation vehicles has been recently reviewed by Tavares et al., (2014). Regarding plant proteins, soy proteins are the predominant choice as wall materials, being those obtained from pulses (such as pea, lentil, or chickpea) and cereal grains (for instance zein or barley protein) the most relevant alternatives (Karaca et al., 2015). Table 2.1 shows some examples of food ingredients that have been encapsulated using proteins.

Table 2.1: Some of the most commonly used proteins for microencapsulation and examples of applications.

Protein	Origin	Examples of Encapsulated Ingredients
Whey proteins	Animal (milk)	α -Linolenic acid (Gómez-Mascaraque and Lopez-Rubio, 2016), lycopene (Pérez-Masiá et al., 2015), cardamom essential oil (Mehyar et al., 2014), folic acid (Pérez-Masiá et al., 2015), probiotics (Gomez-Mascaraque et al., 2016; Lopez-Rubio et al., 2012)
Caseins	Animal (milk)	Garden cress seed oil (Umesha et al., 2015), ω -3 fatty acids (Santhanam et al., 2015), curcumin (Pan et al., 2014), indonesian propolis (Sahlan and Supardi, 2013)
Gelatin	Animal	Green tea polyphenols (Gómez-Mascaraque et al., 2015, 2016e), holy basil essential oil (Sutaphanit and Chitprasert, 2014), sunflower oil (Piacentini et al., 2013)
Soy proteins	Vegetal	Polyphenols and anthocyanins from pomegranate (Robert et al., 2010), ω -3 fatty acids (Santhanam et al., 2015; Gómez-Mascaraque and Lopez-Rubio, 2016)
Pea proteins	Vegetal	Conjugated linoleic acid (Costa et al., 2015), α -tocopherol (Pierucci et al., 2007), oils (Gharsallaoui et al., 2007)
Chickpea proteins	Vegetal	Flaxseed oil (Karaca et al., 2015), folate (Ariyaratna and Nedra Karunaratne, 2015), probiotics (Wang et al., 2014)
Lentil proteins	Vegetal	Flaxseed oil (Karaca et al., 2015), probiotics (Wang et al., 2015)
Zein	Vegetal	Fish oil (Moomand and Lim, 2014), green tea polyphenols

1.3 Carbohydrates Versus Proteins for Specific Applications

As inferred from the previous discussion, both proteins and polysaccharides are generally good candidates for the encapsulation of bioactive ingredients. However, there is no consensus in the literature regarding the best choice among them. Indeed, each particular compound to be protected has unique characteristics and distinct physical–chemical behavior, and thus the choice of an optimal matrix for encapsulation should be done taking into account the properties of both components. Bearing in mind that the choice of wall material will have an impact not only on the encapsulation efficiency and protection of the bioactive ingredient of interest, but also in its release behavior (Dias et al., 2015), the selection of the most adequate encapsulation matrix is of outmost importance in the development of microencapsulation structures. Therefore, the purpose of the following sections is to offer a nonexhaustive summary of some relevant findings regarding the selection of carbohydrate or protein matrices for some of the most relevant groups of bioactive ingredients.

1.3.1 Encapsulation of polyphenols

Natural polyphenols are a group of bioactive molecules that attract a great deal of interest in the food industry due to their great antioxidant activity and their many attributed health benefits. However, these compounds are very unstable and some of them have poor bioavailability and unpleasant tastes (Munin and Edwards-Lévy, 2011). Thus, many scientific works have dealt with microencapsulation of polyphenols to overcome these limitations, using different matrices and processing techniques (Fang and Bhandari, 2010). Robert et al., (2010) reported that the encapsulation efficiency for polyphenols extracted from pomegranate was higher when a soy protein isolate (SPI) rather than a maltodextrin (MD) was used as wall material, while the MD matrix provided greater protection during storage at high temperature (60°C). Higher encapsulation efficiencies and enhanced protection for (–)-epigallocatechin gallate (EGCG, a green tea polyphenol) were also obtained when encapsulated in a protein matrix, namely gelatin, than when entrapped within the carbohydrate chitosan, both by spray-drying (Gómez-Mascaraque et al., 2016b) and electrospraying (Gómez-Mascaraque et al., 2015, 2016a). Similar encapsulation efficiencies as those obtained for chitosan (~85%) had been previously obtained when EGCG was encapsulated within other carbohydrate-based matrices (Rocha et al., 2011). The better results obtained when polyphenols were encapsulated within protein matrices in these works might be related to the fact that these compounds generally exhibit complexing properties toward proteins (Munin and Edwards-Lévy, 2011). Furthermore, the bioaccessibility of EGCG after *in vitro* digestion was reported to be higher when gelatin was used as delivery vehicle as compared to the chitosan carriers (Gómez-Mascaraque et al., 2016a,b).

1.3.2 Encapsulation of oils and flavors

Attempts have been made to determine which encapsulation matrices (or blends thereof) are suitable for lipid encapsulation. For instance, Matsuno and Adachi (1993) proposed a method

that is based on the determination of the drying rate of the emulsions to measure the ability of the wall material to form a dense protective network during spray-drying. However, this method does not take into account the chemical nature of the wall materials, not being able to distinguish between materials with similar drying curves (Pérez-Alonso et al., 2003). Given that many protein and carbohydrate matrices are water soluble or water dispersible, and taking into account that processing in food-grade conditions (i.e., avoiding organic solvents) is imperative in the food industry, the encapsulation of bioactive oils and other lipophilic molecules usually involves the preparation of oil-in-water (o/w) emulsions prior to capsule formation. Thus, the ideal wall material for oil encapsulation should have good emulsifying properties (Jafari et al., 2008). In this sense, the amphiphilic nature of proteins is advantageous as they behave as good emulsifiers (McClements, 2004), allowing improved encapsulation efficiencies and oxidative stabilities (Karaca et al., 2015). For this reason, proteins have been the preferred matrices for the encapsulation of polyunsaturated fatty acids (Gómez-Mascaraque et al., 2016a,b; Moomand and Lim, 2014; Perez et al., 2014) and essential oils (Abd El-Salam and El-Shibiny, 2015; Baranauskienė et al., 2006; Sutaphanit and Chitprasert, 2014) in many works. In general, carbohydrates have low surface activity (Barrow et al., 2013) and usually yield poor retention efficiencies (Jafari et al., 2008), although modification of some polysaccharides has also been proposed to overcome this limitation (Tesch et al., 2002; Trubiano and Lacourse, 1988). Also, some gums, such as gum arabic, contain small protein fractions that provide them with emulsifying properties (Gharsallaoui et al., 2007). Still, the suitability of some polysaccharides as encapsulation matrices for sensitive oils has been questioned. For instance, Kolanowski et al. (2006) reported that encapsulation of fish oil within modified cellulose by spray-drying did not improve its oxidative stability. In another work, Gallardo et al. (2013) claimed that microencapsulation of linseed oil within spray-dried gum arabic did increase its thermal stability, but a great loss of α -linolenic acid was observed upon fortification of bread with these capsules, suggesting limited application of the polysaccharide-based formulation in real food systems. On the other hand, addition of a whey protein isolate to the wall material further improved the thermal stability of the encapsulated oil. More recently, Santhanam et al. (2015) encapsulated fish oil within sodium caseinate, MD, and soy protein and supplemented cakes with the capsules. Their results showed that, among the different matrices, the milk protein was the matrix that yielded the best oxidative stability, which also demonstrated desirable organoleptic characteristics. Indeed, milk proteins are usually smaller and more flexible than vegetal proteins, being more efficiently absorbed at the W/O interface and hence better stabilizing the emulsions (Karaca et al., 2015). Umesha et al. (2015) also proved that milk proteins (specifically sodium caseinate and whey protein concentrate) achieved higher encapsulation efficiencies and provided enhanced oxidative stability to cress seed oil as compared to a carbohydrate-based matrix (blend of MD and gum arabic). Conversely, Al-Ismail et al. (2015) concluded that gum arabic yielded better encapsulation efficiency and oil retention during storage than whey protein isolate for a cardamom essential oil. In summary, although controversy exists among different works, proteins seem to generally yield better results than

polysaccharides as wall materials for the encapsulation of oils, which can be explained in light of their excellent emulsifying properties, forming a protective film at the o/w interface.

1.3.3 Encapsulation of carotenoids

Carotenoids are a group of natural pigments with many attributed health benefits when consumed in sufficient levels, some of which also exhibit provitamin A activity (Qian et al., 2012). Together with polyphenols, carotenoids are the most abundant phytochemicals (Kaulmann et al., 2016). However, they have a very low water solubility and poor chemical stability, resulting in low bioaccessibility upon consumption. Dissolution of carotenoids in edible oils has shown to increase absorption in vitro and in vivo (Ribeiro et al., 2010), and thus o/w emulsions are often prepared before capsule production as a suitable strategy to both increase their bioaccessibility and disperse them in aqueous biopolymer solutions or dispersions. Therefore, some of the previous discussion regarding the encapsulation of bioactive oils could be applied to carotenoids. In this sense, Pérez-Masiá et al. (2015) showed that higher encapsulation efficiencies were obtained when lycopene (previously dissolved in a soy bean oil) was microencapsulated using a whey protein matrix as compared to carbohydrate-based matrices, such as dextran or chitosan. Robert et al. (2003) also obtained higher encapsulation efficiencies for the main carotenoid pigments present in an oleoresin from *rosa mosqueta* upon spray-drying within gelatin capsules than using starch as wall material. Furthermore, gelatin provided a greater protective effect on the main carotenoids, especially on *trans*- β -carotene, than starch. However, information on the bioavailability of these microencapsulated carotenoids during in vitro digestion is scarce (Donhowe and Kong, 2014). Being the bioaccessibility of carotenoids one of their main limitations to exert their bioactivities, this feature should be carefully addressed when selecting a wall material for encapsulation. For instance, alginate has been proposed as a desirable encapsulation matrix for its ability to protect carotenoids from the acidic conditions during gastric digestion, and indeed, Zhang et al. (2016) have recently shown that it effectively protected β -carotene from degradation during digestion. However, the encapsulation within alginate beads caused a reduction in β -carotene bioaccessibility due to insufficient release. The bioaccessibility of carotenoids relies on their incorporation into mixed micelles (Fernández-García et al., 2009; Qian et al., 2012), and research suggests that some polysaccharides, such as alginates or pectins, significantly inhibit micelle formation; and hence carotenoids absorption (Riedl et al., 1999; Yonekura and Nagao, 2009). On the other hand, soluble proteins have also been shown to impede incorporation of carotenoids into the oil phase of the gastric emulsion (Rich et al., 2003), although this limitation might be overcome if carotenoids are previously incorporated in o/w emulsions prior to their encapsulation within protein capsules.

1.3.4 Encapsulation of probiotics

Probiotics are a particular group of bioactive ingredients. They are defined as “live microorganisms which, when consumed in adequate amounts, confer a health benefit to the host” (Pineiro and Stanton, 2007), so they must be alive and metabolically active at the time

of consumption. However, a number of studies report low levels of viable probiotic cells in commercial products (Al-Otaibi, 2009; Lin et al., 2006; Shah et al., 2000; Weese, 2002), emphasizing the need for their microencapsulation. The choice of potential matrices for this group of ingredients is more restricted, as the processing conditions must be adequate to warranty their survival. Moreover, to adequately protect the cells from the acidic environment of the stomach and efficiently deliver them to the intestine, the ideal matrix for their encapsulation should be stable at low pHs and disrupt at pHs above 6 (Gbassi and Vandamme, 2012). For these reasons, one of the preferred matrices for the encapsulation of probiotic bacteria is alginate (Dong et al., 2013). Other anionic polysaccharides with gelling capacity, such as carrageenans have also been used, although their processing at mild temperatures is problematic (Mangione et al., 2003; Yuguchi et al., 2002). Proteins, especially milk proteins, are relevant alternatives to alginate for the microencapsulation of probiotics (El-Salam and El-Shibiny, 2015). Rodrigues et al. (2011) assessed the viability of probiotic bacteria when immobilized in different food-grade polymers and found that whey proteins, together with alginate, were the most adequate materials for most of the strains they tested. Nevertheless, the results were strain-dependent, emphasizing the need for designing the encapsulation system for each specific bioactive ingredient. Lopez-Rubio et al. (2012) also observed that a whey protein concentrate (WPC) exerted enhanced protection to *Bifidobacterium animalis* subsp. *lactis* Bb12 during storage at high relative humidity as compared with a polysaccharide, namely pullulan, both processed by electrospraying. Electrosprayed WPC-based capsules were also used to protect a strain of *Lactobacillus plantarum* (CECT 748 T), which experienced a reduced viability loss during in vitro digestion ($\sim 3 \log \text{CFU g}^{-1}$) (Gomez-Mascaraque et al., 2016). In contrast, alginate-encapsulated *L. plantarum* (strain UFRGS BL011) suffered a viability loss of almost $3 \log \text{CFU mL}^{-1}$ when exposed to simulated gastric juice, and an additional drop of $3 \log \text{CFU mL}^{-1}$ when exposed to simulated intestinal juice (Coghetto et al., 2016). However, these results are not directly comparable, since the protected *L. plantarum* strain is different and the protocols employed for simulation of gastrointestinal conditions also differ. It is worth noting that alginate microcapsules have already been successfully incorporated into real food matrices, such as ice creams, frozen yogurts, and mayonnaise (Corona-Hernandez et al., 2013). The microencapsulation of probiotics has been specifically examined in a number of recent review articles (De Prisco and Mauriello, 2016; El-Salam and El-Shibiny, 2015; Iravani et al., 2015).

1.4 Protein/Carbohydrate Mixtures

As discussed, both polysaccharides and proteins have advantages and limitations as encapsulating agents. Therefore, combinations of them have also been studied as wall materials with the aim of improving the attributes of the individual biopolymers (Bastos et al., 2012; Fernandes et al., 2014; Smilkov et al., 2014). For instance, addition of proteins to MDs, which are considered to be one of the best thermal defenders

(Munin and Edwards-Lévy, 2011) for microencapsulation by spray-drying, allows it to increase the powder recovery of the materials (Muzaffar and Kumar, 2016; Shi et al., 2013), while incorporation of dextrans to proteins as encapsulant material for fish oil increased its oxidative stability (Kagami et al., 2003). Aberkane et al. (2014) observed that the combination of pectin with pea protein for the microencapsulation of PUFA-rich oil enhanced its oxidative stability as compared with the pea protein alone. However, protein-polysaccharide combinations do not always perform better than the individual components. Mehyar et al. (2014) obtained better overall results by encapsulation of cardamom oil within a SPI than using mixtures of the protein with either guar gum or carrageenan. Also, complexation of proteins with polysaccharides (Benichou et al., 2002) represents a widely used strategy for the encapsulation of bioactive ingredients (Gutiérrez et al., 2013; Jain et al., 2015; Nori et al., 2011; Piacentini et al., 2013), being coacervation the second most exploited encapsulation technique in the food industry (Dias et al., 2015). Furthermore, proteins and carbohydrates can be conjugated by dry heating through the Maillard reaction (Martins et al., 2000), and these conjugates have also been recently applied to the encapsulation of food ingredients. Davidov Pardo et al. (2015) developed caseinate–dextran coated zein nanoparticles and used them to encapsulate resveratrol, enhancing its bioaccessibility. Coating the nanoparticles with these Maillard complexes resulted in a greater stability to aggregation under simulated gastrointestinal conditions as compared to those coated with plain caseinate. Ifeduba and Akoh (2016) used the Maillard reaction to crosslink gelatin-gum arabic coacervates to improve the oxidative stability stearidonic acid soybean oil. In addition, Maillard reaction products have been described to exhibit antioxidant properties, but the last stages of the reaction can lead to undesirable color and aromas, and even to toxic compounds in certain cases (Tessier and Niquet, 2007), facts that should be considered when designing this type of carriers.

1.5 Characterization Tools for Studying Release and Stability of Encapsulated Compounds

Evaluating the release and stability of encapsulation systems is crucial to assess their efficiency and to optimize the developed structures in terms of achieving the desired release rates when subjected to particular conditions. Several factors, such as the composition and chemical structure of the encapsulating material (also referred to as “matrix”) and the encapsulated bioactive compound, the interactions established between the bioactive and matrix materials and the morphology of the encapsulating system (size distribution, shape, surface area, porosity, etc.) have a strong impact on the stability of the encapsulating systems and, therefore, they should be taken into account when designing a system for a particular application. Moreover, understanding the mechanisms controlling the release of the encapsulated compound from the matrix when subjected to specific conditions (relative humidity, pH of the medium, temperature, etc.) is essential to maximize the material performance for its intended application.

A wide range of techniques is currently available to investigate the properties and release mechanism in different encapsulating systems. The selection of the characterization methods for a particular encapsulating structure has to be made on the basis of the system composition, release rate and medium. In general, a combination of several techniques, covering from the molecular to the microscale structural levels, is the most efficient approach to thoroughly study the system. The following sections provide a brief overview of the main tools available to characterize encapsulating materials, with special emphasis on the potential of small angle scattering techniques to provide valuable structural information and to exhaustively investigate the release mechanisms under a wide range of conditions.

1.5.1 Microscopy techniques

Imaging techniques based on microscopy instruments are one of the most widely used methods to characterize the structure of encapsulating systems. They may provide qualitative information on the shape, size, and aggregation state of the materials under study. Electron, optical, and laser microscopy methods may be selected depending on the composition of the matrix and the size range of the generated materials.

Although optical microscopy is the most easily accessible and low-cost imaging technique, its main drawback is its relatively low resolution, which is limited by the diffraction of light to about 1000 diameters magnification (Luykx et al., 2008). As an alternative, electron microscopes offer much higher magnifications, which enable the characterization of particles with size ranges from tenths of nm to tenths of μm . Among the electron microscopy techniques, transmission electron (TEM) and scanning electron microscopy (SEM) are the most widely used ones. TEM is a technique that is based on the transmission of a beam of electrons through an ultrathin sample. The major limitation from this technique lays in the tedious sample preparation that is often required. First of all, the samples have to be thin enough to be electron transparent and must be dry to be subjected to the high vacuum present inside the instrument. Additionally, negative staining is often required to increase the contrast between the sample and the background. This involves placing a small amount of the sample on a TEM grid and staining with a solution (such as uranyl acetate), which provides high contrast. To obtain information about the particle internal structure, the sample can be subjected to the freeze-fracture technique. In this case, the sample is placed on the TEM grid, which is then vitrified by rapid freezing and subsequently fractured under constant cooling and vacuum. It is evident that these preparation procedures are likely to have a strong impact on the structure of the sample, especially in the case of biological samples and moisture sensitive materials. SEM presents lower resolution than TEM, but it has a greater depth of view (thus providing more information on the particle surface structure) and the sample preparation is usually less complex. This technique is based on scanning a beam of electrons across the surface of the sample, providing information on the sample surface topography and composition. SEM requires high vacuum and sample conductivity, which is again translated into possible structural modifications induced by the drying and coating processes applied to the sample specimens. As an

alternative, atomic force microscopy (AFM) is a more recent technique in which a physical sharp probe is scanned over a specimen that has been previously immobilized onto a surface. The surface topography of the sample is resolved by determining the reaction of the probe to the forces that the sample imposes on it when scanned. One of the main advantages of AFM, apart from its high resolution (ca. 0.1 nm) is the fact that, unlike SEM, the samples do not need to present a conductive surface. This type of microscopy is, however, not suitable for surfaces that are soft or sticky. In those cases, the sample may need to be dried prior to the analysis.

Although microscopy techniques are routinely used to characterize the structure of encapsulating systems, the results have to be carefully interpreted and any possible structural alterations induced by sample preparations have to be taken into account. Therefore, although microscopy methods may be useful, they should be preferably combined with complementary techniques to ascertain the morphology of the encapsulated system in its native state.

1.5.2 Separation and analytical techniques

Chromatography methods can be routinely used to separate the components in encapsulating systems, that is, the matrix and the encapsulated compound and, subsequently, quantify the amount of the active compound to, for instance, evaluate the encapsulation efficiency. The separation of different constituents in a sample is done on the basis of the different speeds at which distinct compounds travel through a structure (usually a column) containing a specific material (known as stationary phase) when dissolved in a certain fluid (known as mobile phase). The final step after separating the components in the sample consists in the quantification of each component by using a suitable detector, such as an ultraviolet-visible (UV-vis) light absorbance detector, a fluorescence detector, or a diffractometer. The advantage of chromatographic techniques is that they offer a range of possibilities (mobile and stationary phases, type of separation technique, detector type) that can be adjusted depending on the particular composition and properties of the encapsulated system.

Depending on the physical state of the mobile phase, we can differentiate gas-chromatography (GC) and liquid chromatography (LC) techniques. In particular, high-performance liquid chromatography (HPLC) is a widely used technique in the food analysis field. In this particular case, the sample has to be dissolved in a liquid, which is then pressurized and injected into a column containing a stationary phase. Depending on the type of stationary phase, the mechanism for the separation of compounds in the sample will be different, giving rise to a range of chromatographic separation methods. For instance, the separation may occur on the basis of the molecular mass of the different compounds [size exclusion chromatography (SEC) or gel permeation chromatography (GPC)] or the charge (cation or anion exchange chromatography). In addition to HPLC, GC coupled with mass spectrometry (GC-MS) is also one of the most widely used techniques to separate and quantify compounds that can be vaporized without affecting their molecular structure. In this case, after the compounds in the sample travel through the chromatographic column and

are released (with different retention times), the coupled mass spectrometer breaks down the molecular structure of each component into ionized fragments, separates the ions of differing masses, and determines their relative abundance by measuring the intensity of the ion flux.

Some examples of applications of chromatographic techniques include the encapsulation efficiency assessment of gallic acid/cyclodextrin complexes incorporated into electrospun polylactic acid (PLA) nanofibers (Aytac et al., 2016) or resveratrol encapsulated in chitosan–sodium tripolyphosphate microspheres (Cho et al., 2014) by using HPLC coupled with a UV detector, or the use of GC-MS to evaluate the release of essential oils, such thyme essential oil in β -cyclodextrin capsules (del Toro-Sánchez et al., 2010).

Although these techniques are useful to determine the encapsulation efficiency of a certain system, they do not provide information on the structure of the system, the interactions established between the matrix, and the encapsulated compound or the release mechanism-taking place under specific conditions.

1.5.3 Physicochemical characterization methods

A wide range of techniques can be utilized to investigate the structural changes and the mechanisms controlling the release and stability of encapsulated systems. Among the most commonly used methods, spectroscopy and scattering techniques are particularly interesting to study structural changes taking place at the molecular and nano-/microstructural levels. In particular, scattering techniques present an enormous potential, although, to date, their application within this research field is still very limited. This is mainly due to the unfamiliarity of many researchers within the food science field and unawareness of the valuable information that can be extracted from these techniques. However, several works have already demonstrated the great advantages of combining small angle scattering techniques with traditional methods to provide unique insights into the characterization of food systems (Blazek and Gilbert, 2011; Lopez-Rubio and Gilbert, 2009).

1.5.3.1 Spectroscopy techniques

Spectroscopy methods rely in the study of interactions established between a source of radiation (most commonly electromagnetic radiation) and the particles composing a sample and, subsequently, measuring changes in the intensity or frequency of the radiation energy. In particular, nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infrared spectroscopy (FT-IR) are widely used to characterize the molecular structure and molecular interactions between the components in encapsulating systems.

NMR is based on the measurement of the electromagnetic radiation, which is absorbed by an active nucleus, such as ^1H or ^{13}C , at a certain frequency. The absorption of an atomic nucleus is conditioned by the surrounding atoms and, thus, detailed information on the molecular structure of a certain compound can be obtained. This technique can be applied to any kind of

sample, in the solid or solution state, which contains nuclei possessing spin. In the particular case of encapsulating systems, ^1H -NMR and ^{13}C -NMR techniques have been used to prove the formation of stabilizing inclusion complexes between β -cyclodextrins and essential oils (del Toro-Sánchez et al., 2010; Răileanu et al., 2013) or to determine the molecular state of curcuminoids encapsulated in poly(butyl) cyanoacrylate nanoparticles (Mulik et al., 2009). Another interesting fact is that this technique is sensitive to dynamics, that is, it is possible to discern molecular domains with distinct mobility. This can be exploited to determine the level of molecular interaction established between different components in encapsulating materials. For instance, the level of interaction between silica matrices and encapsulated ibuprofen molecules has been estimated based on the ibuprofen molecular mobility deduced from ^{13}C and ^1H spectra (Babonneau et al., 2004). In addition, relaxation experiments, that is, measuring how the signal changes with time, have been applied to study the water–polymer interactions in whey protein/alginate beads containing riboflavin (Wichchukit et al., 2013).

The FT-IR technique, as any other absorption spectroscopy technique, consists on measuring the amount of light absorbed by a sample at different wavelengths. The peculiarity of FT-IR is that instead of using a monochromatic beam of light, the sample is radiated by a beam composed of many wavelengths at once and data are subsequently processed to determine the absorption taking place at each measured wavelength. FT-IR can be utilized to confirm the encapsulation of the active compound into the matrix by identifying characteristic absorption bands. For instance, FT-IR has been used to determine the presence of vitamin B12 and vitamin C encapsulated into different biopolymers by spray-drying (Estevinho et al., 2016), tea tree oil in sodium alginate/chitosan capsules (Chen et al., 2016), xylitol in multicomponent microcapsules obtained by complex coacervation (Santos et al., 2014), and resveratrol in chitosan–sodium tripolyphosphate microspheres (Cho et al., 2014).

1.5.3.2 Scattering techniques: basic principles and application to characterize encapsulated systems

Scattering techniques are based on the analysis of the scattered radiation produced after a source, such as light, X-rays, and neutrons, interacts with the particles present in a sample. They represent a powerful tool to obtain information about the size, shape, and orientation of components in ordered (long-range or crystalline order), but also disordered systems. In practice, the radiation scattered by the sample of interest is typically measured on a two-dimensional detector; for isotropic scatterers, after being radially averaged, the intensity is plotted versus the magnitude of the scattering vector q , which describes the relationship between the incident and the scattered wave vectors, being defined as:

$$q = \frac{4\pi}{\lambda} \sin \theta \quad (2.1)$$

Where θ is half the angle through which the radiation is scattered and λ is the wavelength of the incident radiation.

Combining Eq. (2.1) with Bragg's law ($\lambda = 2d \sin \theta$), one can determine the real-space dimension of the scattering object, through the relationship:

$$d = \frac{2\pi}{q} \quad (2.2)$$

According to this, scattering methods are classified into wide angle and small angle scattering techniques depending on the range of scattering angles (and corresponding length scales) covered. Therefore, whereas wide-angle scattering techniques are used to probe subnanometer dimensions, small angle scattering techniques, are able to cover a size range from 1 nm to several hundreds of nanometers. Coupling small angle with ultrasmall angle scattering techniques it is possible to study larger structures, hence extending the size range up to ca. 10 μm .

It is important to select the proper source of radiation based on the sample composition and structural features to be probed. While X-rays are scattered by the electrons, being SAXS sensitive to variations in electron density, neutrons are scattered by the atomic nuclei and, thus, SANS depends on the nuclear structure of the atom. As a result, whereas X-ray scattering intensity is a function of the atomic number of the scattering elements, in the case of neutron scattering, different isotopes of an element may present significantly different scattering intensity. Conversely, light is scattered by fluctuations in the dielectric constant of a material. It is worth noting that, because the wavelength of visible light is in the order of hundreds of nanometers, light scattering techniques typically probe larger structural features than X-ray and neutron scattering techniques (i.e., low q values are covered in light scattering experiments).

Light scattering techniques are widely used for the characterization of colloidal suspensions, such as those typically found in food systems. Static light scattering (SLC), also known as laser light scattering (LSC), measures particle size based on light scattering intensity, which changes with variation in scattering angle, size of particles, refractive indices of particles, and the medium. SLC measures the average intensity of scattered light arriving at the detector, although actually, the intensity shows fluctuations over short time scales. Moreover, in the case of solutions, the Brownian motion of particles causes fluctuations in the intensity of the scattered light arriving at the detector. Dynamic light scattering (DLS) is based on the measurements of these fluctuations as a function of time. By analyzing these fluctuations through correlation functions, it is possible to extract information regarding the particle size. DLS is commonly used to characterize the particle size of encapsulating systems based on microemulsions (Flanagan et al., 2006; Gaysinsky et al., 2008; Yin et al., 2012; Zhong et al., 2009; Ziani et al., 2012). The main issue of SLC and DLS methods is that highly diluted samples are often required to avoid multiple scattering (i.e., the photons are scattered more than once between the light source and the detector). In addition, to ensure a proper data interpretation, the particles should present a narrow size distribution and they must present the same refractive index (which is not true in the case of multicomponent systems).

The issue of multiple scattering is often avoided when using X-rays and neutrons, because these radiation sources do not scatter as strongly as light. Hence, these techniques are more suitable for the characterization of a wide range of food-based encapsulating systems. The most widely used techniques, classified on the basis of the source of radiation and the covered size ranges, are designed as wide angle and small angle X-ray scattering (WAXS and SAXS) and small angle neutron scattering (SANS). It should be mentioned that WAXS is often regarded as identical to the X-ray diffraction technique (XRD), which is not completely true. While WAXS is conducted in the transmission geometry, XRD may be performed in the reflection or in the transmission geometry. XRD is the most commonly used method to characterize the crystalline structure in materials presenting long-range order. In addition to the previously mentioned techniques, ultrasmall angle scattering techniques (USAXS and USANS) can be utilized to probe larger structural features. Fig. 2.2 shows the relevant size ranges covered by these techniques and complementary methods, as related to the different structural features found in typical encapsulating systems.

The suitability of SAXS or SANS to characterize a particular encapsulating system depends on the scattering length density (SLD) contrast between the sample and the surrounding element materials or between different sample components (i.e., the difference in the scattering intensity of the molecules in the different components). The radiation source should be thus chosen to enhance the SLD contrast between the sample components. Specifically for SANS, the scattering length difference existing between hydrogen ($-0.3742 \times 10^{10} \text{ cm}^{-2}$) and its heavier isotope, deuterium ($0.6671 \times 10^{10} \text{ cm}^{-2}$), is the basis of the contrast variation method. This is an extraordinary valuable approach used to enhance the SLD of hydrogen-containing samples or to selectively match desired components in a multicomponent system by using different $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixtures.

Apart from the nondestructive nature of small angle scattering techniques, they have further advantages, such as enabling the study of representative samples with relatively larger volumes as compared to methods, such as TEM, in which small sample areas of the order of hundreds of square microns are analyzed. On the contrary, the main drawback of small angle scattering techniques is that they are indirect methods and mathematical models have to be applied to describe the experimental scattering curves. These models require prior knowledge of the system to be built and, therefore, it is of outmost importance to combine small angle scattering techniques with complementary methods, such as microscopy and spectroscopy techniques. A multitechnique approach is the most successful path to obtain detailed knowledge of the sample properties based on realistic physical–chemical parameters (Martínez-Sanz et al., 2015, 2016).

As previously mentioned, the potential of SAXS and SANS techniques for the characterization of encapsulating systems has not been fully exploited and very few studies, which are relatively recent, report on the use of these techniques within this particular

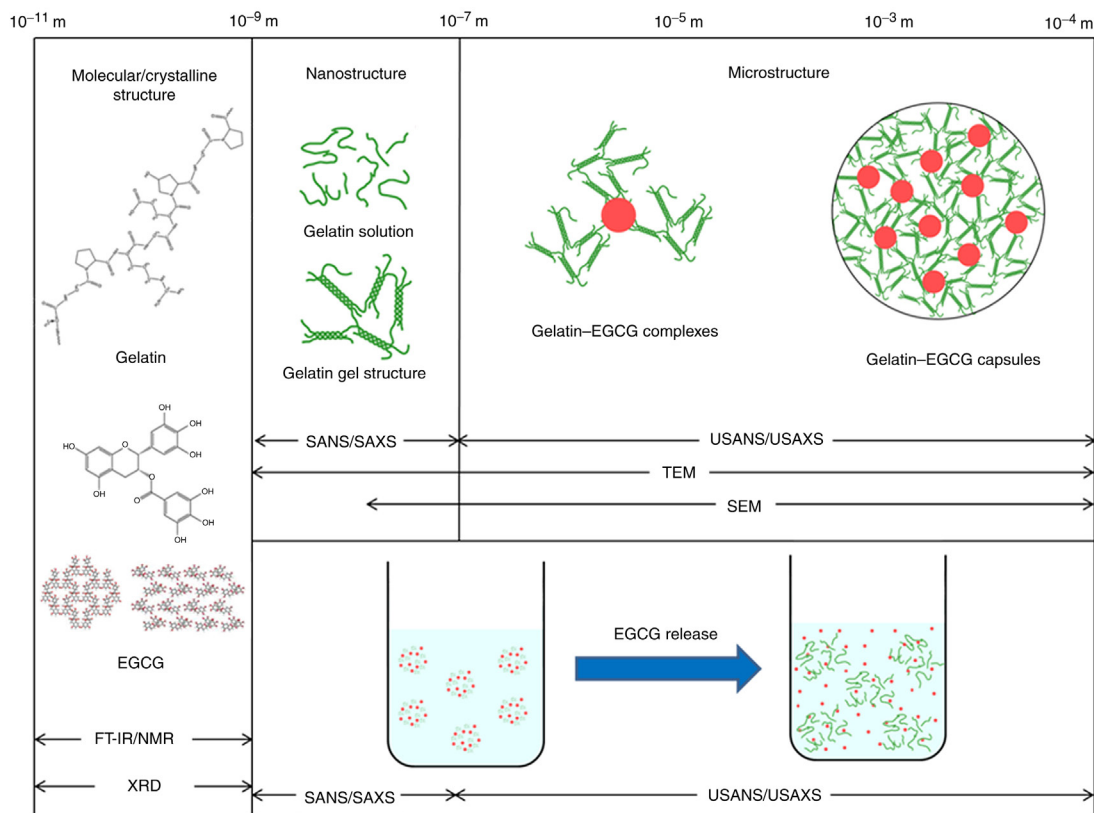


Figure 2.2: The Range of Elastic Scattering Techniques and Corresponding Size Range With Complementary Methods Shown in Relation to Different Structural Levels Relevant to Encapsulation Systems.

The particular case of gelatin capsules loaded with EGCG (Gómez-Mascaraque et al., 2015) is provided as an example of encapsulation system. Adapted from Lopez-Rubio, A., Gilbert, E.P., 2009. Neutron scattering: a natural tool for food science and technology research. *Trends Food Sci. Technol.* 20(11–12), 576–586; Martínez-Sanz, M., Gidley, M.J., Gilbert, E.P., 2015. Application of X-ray and neutron small angle scattering techniques to study the hierarchical structure of plant cell walls: a review. *Carbohydr. Polym.* 125, 120–134.

research field. Nevertheless, the existing works highlight the adequacy to investigate the release mechanisms and stability of encapsulating materials. As an example, some of the most relevant works are summarized later.

Gerelli et al. (2008) reported on the structural characterization of soybean lecithin/chitosan nanoparticles loaded with a lipophilic positively charged drug used in breast cancer therapy by combining cryo-TEM, static light scattering, and SAXS and SANS techniques. By applying proper models to describe the experimental scattering data, it was possible to determine the structure of the encapsulating vesicles, as well as the interactions established

between the matrix and the encapsulated drug. The unilamellar structure of liposomes loaded with antibiotics has also been investigated by means of SAXS characterization (Colzi et al., 2015). The internal structure of poly(lactide-*co*-glucolide)-*block*-poly(ethylene glycol) (PLGA-PEG) nanoparticles, commonly used as drug carrier systems, was resolved by finding a proper model (consisting of fractal structures with polydisperse spherical building blocks) to simultaneously fit the SANS experimental data from the nanoparticles dispersed in two different H₂O/D₂O mixtures (Yang et al., 2015). This allowed identifying the release mechanism of a hydrophilic anticancer drug, such as carboplatin, when incorporated into the PLGA-PEG nanoparticles. The distinct SLD contrast generated when using neutrons and X-rays was exploited to characterize microemulsions embedded in alginate hydrogels (Josef et al., 2013). Whereas X-rays provided a low SLD contrast between the microemulsion and the alginate gel (thus probing the structure of the whole system), the use of neutrons enhanced the SLD contrast between the two phases, hence highlighting the structure of the microemulsion droplets embedded within the gel. In another study, fractal analysis and evaluation of the particle distance distribution function from SAXS experiments evidenced the structural changes undergone by bovine serum albumin when combined with chitosan and poly(sodium-4-styrene) sulphonate to produce core-shell nanoparticles loaded with ibuprofen for controlled release of the drug (Varga et al., 2014). Liu et al. (2011) investigated the release of an enzyme encapsulated in freeze-dried chitosan/xanthan gum hydrogels and observed that the release was triggered by lowering the pH of the medium. On the other hand, the addition of montmorillonite (MMT) nanoclay into the hydrogels was seen to stabilize the system, lowering the enzyme release rate. The use of WAXS and SAXS was crucial to explaining the stabilizing effect of MMT. Although WAXS evidenced that a high degree of MMT intercalation within the chitosan/xanthan gum blend was achieved, SAXS showed that the incorporation of MMT reduced the structural changes undergone by the hydrogels when the pH of the medium was modified. The encapsulation of a vitamin into spray-dried protein-stabilized emulsions was evaluated and the vitamin stability was linked to structural changes by combining light scattering and differential scanning calorimetry with SAXS characterization (Relkin et al., 2014). The formation of lamellar structures as a result of the packing of the fatty phase, evidenced by the presence of a Bragg peak in the SAXS patterns, was seen to reduce the vitamin degradation.

2 Nanostructured Biopolymer Layers for Food Packaging

2.1 Improving Barrier Properties of Packaging Materials

Due to the severe environmental pollution caused by plastic food packaging, there has been a growing amount of interest in the production of biodegradable films, which, at least, partially replace nonrenewable conventional petroleum-based packaging materials (Faruk et al., 2012; Jiménez et al., 2012). Among the most widely researched biodegradable thermoplastics, sustainable biopolymers for monolayer packaging applications are starch,

polyhydroxyalkanoates (PHA), and PLA. Specifically, starch and PLA biopolymers are, undoubtedly, the most interesting families of biodegradable materials, as they have become commercially available, being produced in a large industrial scale. Of particular interest in food packaging is the case of PLA due to its excellent transparency and relatively good water resistance. However, as already commented, the main challenge for these specific biomaterials is to improve their properties so that they perform (in terms of barrier and thermal properties) like polyethylene terephthalate (PET).

Biopolymer materials extracted from biomass resources, such as proteins (e.g., zein, wheat gluten), polysaccharides (e.g., starch, chitosan), and lipids (e.g., waxes) have excellent potential as gas and aroma barriers in dry state but they have very strong water sensitivity arising from their hydrophilic nature, which leads to a strong plasticization and the subsequent deterioration of film properties as relative humidity and water sorption in the material increase. In general, one of the main disadvantages of these biopolymers is that they are permeable, in more or less extent, to low molecular weight compounds (i.e., water vapor, oxygen, and aroma compounds), which can compromise packaged food quality and safety. In the particular case of starch, although native starch is not an inherently thermoplastic material, it can be processed like conventional polymers, giving raise to what is known as thermoplastic starch (TPS), after some specific processing steps. However, TPS shows several drawbacks that reduce their applicability as packaging material, such as their highly hydrophilic character, limited mechanical properties, and the retrogradation phenomenon that occurs during ageing (Jiménez et al., 2012). To avoid this problem, several studies have been focused on the optimization of the physicochemical properties of packages and the minimization of food-package interactions, such as gas or aroma migration, by developing nanocomposites and multilayer food packaging structures.

One of the most used approaches to improve the performance of packaging materials is the use of blends (e.g., plasticizers, other biopolymers) or the addition of nanomaterials (e.g., nanoclays) to achieve the desired properties (Bordes et al., 2009). However, it is well known that the most efficient structure to constitute high barrier materials using two element components is for them to be disposed in a layered form (Fabra et al., 2013). This methodology has been widely used in synthetic polymers but it has been barely developed in biodegradable food packaging systems because it is difficult to assemble materials thermodynamically immiscible without the addition of synthetic adhesives. In fact, one of the main challenges in the development of multilayer biobased and biodegradable films is to achieve good adhesion between layers. Interestingly, both electrospinning and layer-by-layer (LbL) assembly can at the same time be used to enhance physicochemical and barrier properties of packaging materials and for the entrapment of functional compounds to develop active packaging structures (Fabra et al., 2013, 2015, 2016d,b). In this regard, Fabra et al. (2014a,b, 2015) have successfully developed hydrophobic coatings or interlayers with bioadhesive properties to improve the barrier and functional performance of biodegradable

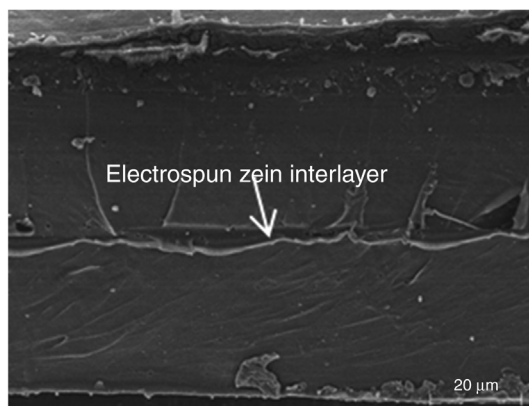


Figure 2.3: Sem Image of the Cross-Section Corresponding to a Multilayer Film Composed of Two PLA Outer Layers and an Electrospun Zein Interlayer.

polymers combining biomass-derived nanocomposites and the electrospinning process. [Fig. 2.3](#) shows an example of the microstructure reached in multilayer films obtained with PLA (outer layer) and electrospun zein interlayer.

Recently, it has been reported that electrospun zein interlayers have the potential to generate multilayered biopolymeric structures with improved barrier properties in PLA ([Busolo et al., 2009](#)) and PHA-based films ([Fabra et al., 2013, 2014a,b](#)) with minimum changes in the optical properties, which could be ascribed to the light scattering generated by the arrangement of the biopolymeric components distributed throughout the inner layer. Specifically, [Fabra et al. \(2014b\)](#) demonstrated that biopolyester-based multilayers prepared with the electrospun zein interlayers significantly improved oxygen permeability values (up to 70%) of PLA and PHA biopolymers, although the water vapor barrier properties depended on the biopolymer used as outer layers. As opposed to the PHA's materials performance, PLA did not improve the water vapor permeability values of these films. However, not only the nature of the outer layers but also the morphology, thickness, and the inherent barrier of the electrospun interlayer materials determine the oxygen and water vapor barrier properties of the overall multilayer. For instance, [Fabra et al. \(2014a\)](#) reported that fibrillar structures obtained for zein and pullulan electrospun interlayers significantly contributed to improve barrier performance of biopolyester-based multilayer systems whereas bead microstructures obtained by electrospinning a whey protein isolate did not have an impact on it.

The methodology already described has also been proven to avoid the moisture uptake of hydrophilic-based thermoplastic materials (i.e., wheat gluten or starch). To this end, biopolyester coatings have been directly electrospun onto both sides of hydrophilic wheat gluten or starch based films ([Fabra et al., 2015](#)). As opposed to the earlier mentioned structures obtained by using electrospun bioadhesives, the structures developed by sandwiching thermoplastic proteins and polysaccharides-based films with electrospun

hydrophobic coatings were not high barrier materials and they could be used for some fresh produce (e.g., mushroom, strawberry) with high respiration rates that require packaging films with high oxygen and carbon dioxide permeability values. In this type of multilayer structures, proteins and polysaccharides have to be first plasticized to become thermoplastic and form a continuous film, a fact that made them suitable in terms of barrier properties, for fulfilling the packaging requirements of fresh produce. However, water and oxygen permeability values of the neat thermoplastic wheat gluten or TPS films could be detrimentally altered by the presence of plasticizers and these biopolymers could be disintegrated or even dissolved at high relative humidity conditions, limiting their real application. To overcome this issue, electrospun hydrophobic coatings were incorporated as outer layers, avoiding swelling problems of the plasticized wheat gluten or starch-based film. In this regard, [Fabra et al. \(2016c\)](#) have observed that barrier properties of the developed multilayer systems can be tailored by using different biodegradable electrospun coatings (such as PLA, polycaprolactone, PCL, or PHA) and by modulating the thickness of the outer layers. In fact, they have found a positive linear relationship between the amount of the electrospun coatings deposited onto both sides of a thermoplastic cornstarch film and the thickness of the electrospun biopolyester coating. Interestingly, the addition of electrospun coatings led to an exponential oxygen and water vapor permeability drop as the amount of the electrospun coating increased.

Another interesting approach to enhance the overall functionalities of TPS-based film, with special focus on their barrier performance, is the development of multilayer systems in which the inner layer is a nanobiocomposite starch-based film containing bacterial cellulose nanowhiskers (BCNW). Thereafter, the optimized nanobiocomposites were successfully hydrophobized by coating them with electrospun PHA or electrospun PHA-BCNW fibers. To this end, hybrid electrospun PHA fibers reinforced with highly dispersed crystalline BCNW in solutions concentrations up to 15 wt.% were directly electrospun onto both sides of starch based nanobiocomposites containing 15 wt.% BCNW. Results showed that the incorporation of BCNW in one of the layers of a multilayer system enhanced oxygen barrier properties to a great extent ([Fabra et al., 2016e](#)).

Therefore, by means of the technology here proposed, one can tailor the barrier properties of food-packaging materials according to the final intended use. Interestingly, another advantage arising from this procedure is that this technology also offers the possibility to develop active and bioactive packaging multilayer structures producing simultaneously interlayers or coatings with encapsulation performance of the active/bioactive compounds thus preserving their functional properties. In this sense, [Fabra et al. \(2016d\)](#) have developed active multilayer food-packaging materials by means of electrospinning. Specifically, α -tocopherol, as a model antioxidant, was encapsulated in three different protein-based matrices (whey protein isolate, SPI, and zein), which were directly applied as coatings onto a thermoplastic wheat gluten film. The antioxidant activity of the active compound was preserved during the encapsulation

process (up to 95%) and it was seen that the electrospun shell materials were able to protect the antioxidant from degradation during a typical sterilization process.

Another technique with great potential in the development of nanoscopically structured new materials is the sequential LbL adsorption of polymer of solid substrates (Pinheiro et al., 2012; Weis et al., 2006). By means of this procedure, several biopolymers with opposite charges can be used to produce nanolayered films assembled through LbL. The LbL technique is quite simple and enables using a wide range of materials (e.g., proteins, polysaccharides, lipids, and nanoparticles). These materials are able to interact either by electrostatic interactions, hydrogen bonding, covalent bonds, complementary base pairing, or hydrophobic bonding. For instance, two polysaccharides with opposite charges, chitosan and sodium alginate, were deposited onto aminolyzed/charged PET (A/C PET) and the resulting multilayer nanofilm showed lower water vapor permeability that could be expected for the materials with which the film was built (Carneiro-da-Cunha et al., 2010). The use of the LbL technique has also been studied for the development of active coatings. In this line, Fabra et al. (2015) developed LbL films by alternating layers of alginate and zein/carvacrol nanocapsules with improved barrier properties and antifungal activity against *Alternaria* sp.

2.2 Imparting Antibacterial and Antiviral Activity

2.2.1 Antibacterial polymers

The globally increasing demand for minimally processed, easily-prepared, and ready-to-eat “fresh” food products has encouraged manufacturers to develop new packaging technologies. These demands are strongly influenced by the market trends, which together with globalization of food trade and distribution from centralized processing facilities, are the driving forces for continuous development and upgrade of food packaging (Appendini and Hotchkiss, 2002; Ozdemir and Floros, 2004).

Active packaging is an innovative solution to meet the continuous changes in current consumer demands and market trends because it extends shelf life and improves safety of the food by scavenging of oxygen, moisture, or ethylene, and by promoting emission of ethanol, flavors, and antimicrobial agents (López-Rubio et al., 2004). In particular, antimicrobial packaging is attracting increased attention from the food and packaging industry, because the use of preservative packaging films offers several advantages compared with the direct addition of preservatives into food products (Suppakul et al., 2003). The incorporation of antimicrobial agents into polymeric films allows industry to combine the preservative functions of antimicrobials with the protective functions of the preexisting packaging concepts (Appendini and Hotchkiss, 2002). Antimicrobial activity can be achieved by including pads containing volatile antimicrobial agents into packages, incorporating antimicrobial agents directly into polymers, coating antimicrobials onto polymer surfaces, immobilizing antimicrobials by chemical grafting, or using polymers that are antimicrobial by themselves (Malhotra et al., 2015).

In this context, the application of engineered nanomaterials is considered a promising tool to improve the functionality of biopolymers used in antimicrobial food packaging and antimicrobial food contact surfaces. It has been demonstrated that the high surface-to-volume ratio of nanostructured materials enhance the antimicrobial activity at low concentrations and, additionally, provides exceptional chemical and physical properties as an additive for material for the UV-shielding, deodorizing, antiseptic, and so on. In fact, antibacterial nanoparticles composed of metals, metal oxides, metal salts, metal hydroxides, organic nanocarriers loaded with antibacterial agents, hybrid materials, and polymers exhibiting antimicrobial properties themselves have been recently developed, being the metal nanoparticles of zinc oxide and silver, the most common nanoparticles used (Mauriello, 2016; Moritz and Geszke-Moritz, 2013).

Metal nanoparticles can be used as antimicrobial agents because they have numerous advantages over other antimicrobials. Their broad spectrum against foodborne pathogens (even at very low concentration) (Lara et al., 2010; Nocchetti et al., 2013) and their negligible toxicity toward human cells at the same concentration ranges, as well as their improved stability and low volatility in comparison with organic antimicrobial agents (Dutta et al., 2012), make them a promising materials for food packaging and food contact surfaces applications. However, the main challenge today in producing this kind of antimicrobial is the synthesis of stable nanoparticles in a polymer solution, as their antimicrobial effectiveness not only depends on their size but also on their size distribution and agglomeration state (Castro-Mayorga et al., 2016b). In this regard, biopolymers have emerged as suitable platforms for metal nanoparticles because they can act as carriers for stabilizing metal nanoparticles against agglomeration and simultaneously constitute a biodegradable active material.

The effectiveness of nanoclays and nanosilver as antimicrobial agents and, hence, their possible use in active packaging systems, has been demonstrated in various in vitro experiments as summarized by Kuorwel et al. (2015). In the case of silver, several research works have investigated the effect of incorporating silver fillers and nanofillers into different biopolymer matrices, such as poly(vinyl alcohol) (Fortunati et al., 2013; Krklješ et al., 2007; Mbhele et al., 2003) PLA (Martínez-Abad et al., 2014) but these silver particles were not synthesized and stabilized into the matrices, thus causing significant changes in the optical properties and reducing the antimicrobial efficiency. In this way, the pioneering studies made by Castro-Mayorga et al. (2014) examined not only the role of a PHA as stabilizing agent for the in situ synthesis of silver nanoparticles (Castro-Mayorga et al., 2014) but also the use of this material as masterbatch for the preparation of active nanocomposites films by direct blending with a commercial poly(3-hydroxybutyrate-co-3-hydroxyvalerate). The so obtained films led to a surprising oxygen permeability drop of ca. 56% compared to the neat polymer and had a high and prolonged bactericidal activity against two of the most common food borne pathogens, *Salmonella enterica* and *Listeria monocytogenes* (Castro-Mayorga et al., 2016a).

However, the highest potential of metal nanoparticles as antimicrobial agents can hardly be achieved in most cases because they have low solubility/compatibility with the polymers, leading to the agglomeration of nanoparticles, and the prepared antibacterial polymeric materials present low surface-to-mass ratios. To solve this problem, the metal nanoparticles can be alternatively preincorporated into submicro- or nanofibers by means of electrospinning to generate masterbatches, which are subsequently melt mixed with polymer pellets, or even better, used as active coatings over polymer surfaces (Amna et al., 2014). The incorporation of metal nanoparticles in electrospun fibers has enabled the development of novel materials with useful features, such as fibrous membranes with antibacterial properties for water filtration (Botes and Cloete, 2010), protective clothing (Pant et al., 2011), wound dressings, implant materials, or tissue engineering (Navalakhe and Nandedkar, 2007). Specifically, in the active food packaging area, the electrospinning technique successfully avoids the agglomeration of metal nanoparticles and greatly increases their antimicrobial activity. For instance, ZnO nanoparticles showed a more effective and prolonged biocide activity against foodborne pathogens when they were applied as a coating of annealed electrospun PHA/ZnO fiber mat (Fig. 2.4A) over a compression molded PHA film (Fig. 2.4B) than their counterparts prepared by melt-mixing. This indicated that the biocide potential of metal nanoparticles could be more efficiently exploited when they were incorporated in electrospun coatings, as this processing technique improved the dispersion and distribution of the nanoparticles within the polymer matrix (Castro-Mayorga et al. 2016a).

Beside the metal nanoparticles, the incorporation of other natural compounds, such as essential oils by electrospinning is being considered an interesting option for active food packaging, especially taking into account that these compounds are thermally sensitive

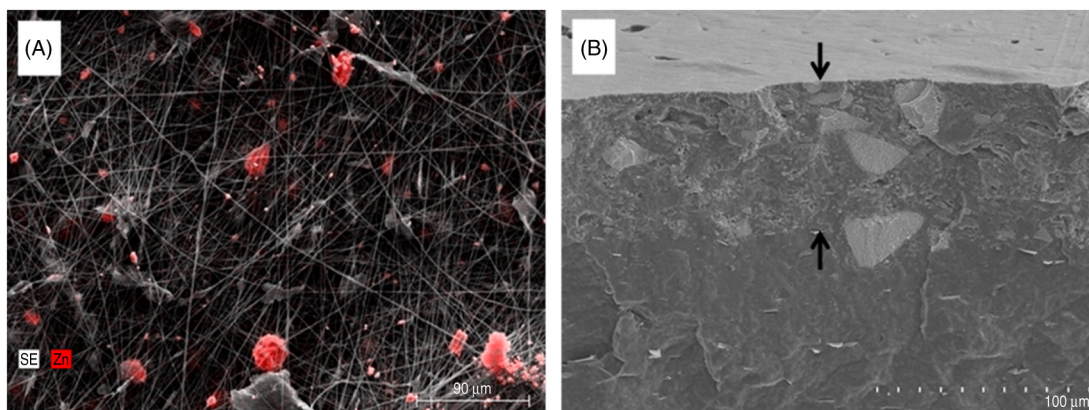


Figure 2.4: Antimicrobial PHA/ZnO Material.

(A) Elemental mapping of electrospun PHA/ZnO fibers made by EDAX analysis from SEM images [Zn in light gray (red in the web version)]. (B) Bilayer PHA system showing the fiber mat PHA/ZnO coating onto PHA film. The arrows indicate the thickness of the coating.

and, thus, cannot be directly incorporated during typical processing methods used for polymeric materials. For example, [Wen et al. \(2016a,b\)](#) carried out in vitro studies with cinnamon essential oil, β -cyclodextrin, and polyvinyl alcohol (PVA) nanofibrous films, which demonstrated an excellent antimicrobial activity against Gram-positive and Gram-negative bacteria. In addition, they also proved that these nanofibrous films were able to prolong the shelf life of strawberries and pork. Likewise, [Munteanu et al. \(2014\)](#) incorporated allyl isothiocyanate (AITC) and β -cyclodextrin into PVA nanofibers via electrospinning and demonstrated that the evaporation of AITC was hindered by cyclodextrin inclusion complexation and thus, their antimicrobial effect against *Escherichia coli* and *Staphylococcus aureus* was enhanced.

Additionally, natural polymers, such as chitosan and cellulose, have also been used to make nanostructured antimicrobial materials. The original antimicrobial chitosan was used by [Torres-Giner et al. \(2008\)](#) for the preparation of active fiber using electrospinning and by [Cárdenas et al. \(2009\)](#) as polymer matrix for the incorporation of nanocopper, which exhibited antimicrobial activity against *S. aureus*, and *Salmonella typhimurium* with a 3–4 log CFU mL⁻¹ bacterial reduction. A cellulose-derived polymer, that is, methylcellulose has been utilized to yield good mechanical properties and barrier properties but also to synthesize silver nanoparticles with high antimicrobial activity ([Maity et al., 2012](#)). Another interesting application was one developed by [Munteanu et al. \(2014\)](#), who combined the antimicrobial properties of silver nanoparticles with the antioxidant activity of vitamin E within multifunctional electrospun PLA/AgNP/vitamin E nanofibers, which may be quite applicable for the fabrication of active packaging structures for fruits and juices.

2.2.2 Polymers with virucide activity

Enteric viruses are those human viruses that are primarily transmitted by the fecal–oral route, either by person-to-person contact or by ingestion of contaminated food or water, although they may also be shed in vomitus. Food may be contaminated by enteric viruses during all stages of the food supply chain, and transmission can occur by consumption of food contaminated during the production process (primary production, or during further processing), or contaminated by infected food handlers.

Data on viral foodborne diseases are still fragmented, and epidemiological studies have focused either on particular countries or on particular pathogens. Epidemiological evidence indicates that enteric viruses, in particular human noroviruses (NoV), which cause acute gastroenteritis, are the leading causes of foodborne illnesses in industrialized countries ([CDC, 2013](#); [EFSA and ECDC, 2015](#)) while hepatitis A virus (HAV) has recently been considered as a reemerging foodborne public health threat in Europe due to the number of foodborne outbreaks associated with imported foods ([Sprenger, 2014](#)). Recently, the World Health Organization estimates the global burden of foodborne diseases, reporting that infectious agents that cause diarrhoeal diseases accounted for the vast majority (550 million cases per year), in particular norovirus

(120 million cases per year) (WHO, 2015). In the EU, foodborne viruses were identified as the most frequently detected causative agent of foodborne outbreaks in 2014, accounting for 20.41% of the reported outbreaks (EFSA and ECDC, 2015). Moreover, the cost of foodborne illness in the USA is estimated to be around 3 billion dollars a year (Scharff, 2012). Other enteric viruses, including sapoviruses, rotaviruses, coronavirus, astroviruses, and hepatitis E virus (HEV), are not frequent causes of foodborne disease but can occasionally be transmitted by contaminated foods.

Although there is an increasing awareness of the importance of foodborne diseases caused by enteric viruses, few studies have confronted the task of evaluating materials with antiviral activity against enteric viruses. In a recent innovative study, an active renewable packaging material with virucide properties was synthesized by the incorporation of silver ions into polylactide acid films. These films showed strong antiviral activity on feline calicivirus (FCV), a norovirus surrogate, using the Japanese industrial standard (JIS Z 2801) (Martínez-Abad et al. 2013). When films were applied to vegetables, antiviral activity was very much dependent on the food type and temperature. Likewise, Bright et al. (2009) evaluated the antiviral activity of active packaging, reporting that FCV titers were reduced by 5 log₁₀ when in contact with plastic coupons impregnated with 10% silver–copper zeolites. Moreover, the antiviral activity of adding grape seed extract (GSE), green tea extract (GTE), or cinnamaldehyde (CNMA) into films has also been reported. Amankwaah (2013) developed chitosan edible films incorporated with GTE or GSE, which showed significantly antiviral activity against murine norovirus (MNV).

Fabra et al. (2016a) showed that the incorporation of an active electrospun interlayer based on zein and 9.7% of CNMA to a polyhydroxybutyrate packaging material (PHB) in a multilayer form completely inactivated FCV according to ISO 22196:2011, while MNV titers were reduced by 2.75 log₁₀. When the developed multilayer films were evaluated after 1 month of preparation or at 25°C, the antiviral activity was reduced as compared to freshly prepared multilayer films evaluated at 37°C.

These studies show the excellent potential of polymers with virucide activity for food contact applications, as well as for active packaging technologies to maintain or extend food quality and safety.

3 Conclusions and Outlook

Summarizing, this chapter, which includes an overview of relevant developments on biopolymer-based materials for encapsulation and packaging applications in the food area, shows the potential of nanostructuring these biopolymers for advanced applications. Not only nanostructuring the biopolymers, but also combining them with nanoparticles can serve as strategies for the development of matrices for bioactive protection and controlled delivery,

interlayers of natural adhesives to improve barrier properties of multilayer packaging structures, coatings to impart hydrophobicity to hydrophilic biopolymers or as tools to develop novel active and bioactive packaging structures. Many of these novel concepts are just beginning to be explored and the interactions between the biopolymers and the active and bioactive substances and/or nanoparticles, which are key to defining the final properties need to be thoroughly studied for their rational design depending on the intended final application. Therefore, it is foreseen that biopolymer nanostructuring will be an increasingly attractive field of materials science, which is expected to contribute significantly toward improving food quality and safety.

Acknowledgments

This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) (RYC-2012-09950, AGL2015-63855-C2-1 and INIA grant RTA2014-00024-C04-03). GS and MJF were supported by the “Ramón y Cajal” Young Investigator from the MINECO. JLC-M was supported by the Administrative Department of Science, Technology and Innovation (Colciencias) of the Colombian Government.

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