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Chapter 9. Uptake and Digestion

Amelia Torcello-Gómez* and Alan R. Mackie

School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, United Kingdom.

Email: M.A.TorcelloGomez@leeds.ac.uk , a.r.mackie@leeds.ac.uk

Tel: +44 (0)113 343 1411, +44 (0)113 343 2959

*Corresponding author

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Abstract

This chapter reviews the gastrointestinal (GI) behavior of the bio-nanosystems introduced in Chapters 2–5, as carriers of bioactive compounds *in vitro* or *in vivo*. For that purpose, an introduction to the main barriers that any food formulation and, in particular, nanocarriers must face when entering the mouth, stomach and small intestine before absorption, is given, as well as the most common *in vitro*, *ex vivo* and *in vivo* assays of digestion and uptake. Next, we focus on the performance of developed bio-based nanocarriers encapsulating bioactive compounds, paying attention to the digestion process within the GI tract, cargo release, bioaccessibility, interactions with intestinal mucosa, uptake or absorption, and bioavailability. The chapter also provides some future perspectives on the interaction between bio-nanosystems and food.

Keywords: digestion, bioactive, gastrointestinal, bioaccessibility, intestinal mucosa, food interactions

9.1. Introduction

The term uptake refers to intestinal absorption and can be defined as the fraction of an oral dose that is absorbed through the intestinal walls. This differs from the term bioavailability, which refers to the fraction of a dose that is available at the site of action in the body, often interpreted as entering the bloodstream (Acosta 2009). Therefore, uptake precedes oral bioavailability, although not all the fraction absorbed in the intestine may become bioavailable since other processes are involved in the absorption of nutrients. Uptake also relates to the term bioaccessibility, which is the fraction of a dose that is potentially available for absorption or uptake after digestion. The gastrointestinal (GI) tract contains several major barriers that need to be overcome for optimal absorption (uptake) and bioavailability of bioactive compounds. These include the potentially low pH of the stomach, the sharply varying pH values, digestive enzymes, the mucus layer that lines the GI tract, and the intestinal epithelium (Kalantzi et al. 2006).

Encapsulation in nanoformulations improves the solubility and stability of bioactive compounds and may partially protect them from the degradative environment in the stomach and small intestine, and achieve controlled release at site of absorption; but GI motility significantly limits their retention. Therefore, the bioavailability of bioactive compounds loaded into nanosystems still requires improvement, requiring further research on the interactions between nanocarriers and GI tract to achieve optimized absorption of bioactive compounds in nutraceuticals (Katouzian and Jafari 2016).

9.2. Gastrointestinal barriers during digestion and absorption

The pH environment in the GI tract is indeed complicated. The normal pH range for the stomach under fasted conditions is between 1.0 and 2.5 due to the presence of hydrochloric acid. However, this can rise to above pH 5 in the fed state, depending on the properties of the meal. The pH value in the small intestine is between 6.0 and 7.0, whereas the mean pH in the distal ileum and in the body fluid at intercellular spaces between enterocytes is about 7.4 (Evans et al. 1988). This pH variation can be even more complex due to the buffering capacity of food in the fed state, making it difficult to keep nanocarrier integrity throughout the entirety of the GI tract. Furthermore, nanocarriers may be susceptible to degradation by digestive enzymes: proteases (mainly pepsin in the stomach, and trypsin, chymotrypsin and elastase in the small intestine), lipases (mainly gastric lipase in the stomach and pancreatic lipases in the small intestine) and amylases (salivary amylase in the mouth and pancreatic amylase in the small intestine). Along with pH variations and enzymes, there are endogenous surface-active components, such as low concentrations of phospholipids in the stomach or in greater concentrations in the small intestine where they are mixed with bile acids. Although these biosurfactants help to emulsify fats and oils and solubilize digestion products into mixed micelles (Maldonado-Valderrama et al. 2011), they can also contribute to the colloidal destabilisation of nanocarriers within the GI tract (Jodar-Reyes et al. 2010).

Nanocarriers must retain their cargo of bioactive compounds and reach the small intestine to be absorbed. Once there, the nanocarriers must overcome the barrier of the mucus layer lining the surface of the GI tract. Mucus is secreted by goblet cells and submucosal glands and is composed of large anionic glycoproteins, predominantly of the mucin family, forming an entangled and crosslinked network. In the small intestine the primary secreted mucin is MUC2. However, the composition and average thickness of the mucus layer varies throughout the GI tract, being as thick as 170 μm in the stomach to 10 μm in the ileum (Lai, Wang, and Hanes

2009) for optimized nutrient absorption. One major problem of this barrier is the quick mucus clearance and the high enterocyte turnover (from 2 to 5 days in human small intestine) that can also clear nanocarriers entrapped in the loosely adherent outer mucus layer (Ensign, Cone, and Hanes 2012). In addition, extracellular DNA from shed epithelial cells can significantly contribute to the microrheological and permeability properties of small intestine mucus (Macierzanka et al. 2014). The mucus layer is a viscous hydrated matrix (Macierzanka et al. 2011), that reduces the shear effect from the movement of GI fluids creating a so called unstirred layer. Therefore, if the nanosystems exhibit mucoadhesive properties and can bind to the mucus layer, they will not diffuse to the enterocytes for absorption. However, if they are free to diffuse through the low-viscosity pores/channels in the mucus network, they can be absorbed. Negatively charged hydrophilic particle surfaces exhibit limited interactions with mucus, conferring rapid diffusion through the mucus layer, but is detrimental to membrane permeation and entry into the epithelial cell (Lundquist and Artursson 2016).

The secreted mucus layer is linked to the enterocytes through the membrane-bound mucins comprising the glycocalyx. The glycocalyx covers the microvilli that crenelate the upper surface of each epithelial cell providing a large area for nutrient absorption. Here, the transport through the cell membrane in the epithelial layer will depend on the size and chemical compatibilities between the surface of the nanocarriers and intestinal epithelium. The presence of tight junctions in the interstitial space between epithelial cells (0.3-1 nm) limits passive diffusion of nanocarriers *via* paracellular route (Pade and Stavchansky 1997). These are mostly transported by transcellular route, whereas hydrophilic, polar solutes diffuse through the paracellular route (Norris, Puri, and Sinko 1998). The transcellular route involves the uptake by epithelial cells in a process called transcytosis, either by enterocytes, which represent 90-95 % of the intestinal epithelial cells, or by M cells (microfold cells) which are located in the Peyer's patches primarily in the distal intestine. M-cells are involved in endocytosis of

macromolecules and microbes that can be potentially antigenic. Transcellular uptake can take place *via* two modes of transport: active and passive. Active transport occurs through specific transporter channels on the surface of the epithelial cell that use the cell's own energy and is regulated by the cell in such a way that a certain level of nutrients and minerals are maintained in the blood. Any excess of these substances is accumulated in tissue or excreted and additional doses are not absorbed via active mechanism. Passive transport occurs by simple diffusion and is controlled by differences in activity of the specific nutrient across the epithelial tissue, defined by the concentration times the activity coefficient. The activity coefficient is inversely proportional to the solubility of the nutrient. Therefore, more hydrophilic compounds tend to have low permeability and transport by means of active mechanism, whereas more hydrophobic compounds are very permeable and absorb via passive and active transport. Passive and active transport apply to both enterocyte and M cells. Although M cells are more permeable, they only represent less than 1 % of the total intestinal area, making selective delivery to these cells more difficult (Hussain, Jaitley, and Florence 2001). If the direct uptake of the nanocarriers is not possible, then they should at least release the encapsulated bioactive compound in the small intestine in a sustained manner. This can be attained with the controlled degradation of the carrier by digestive enzymes or via pH-sensitive materials such as polymers (e.g. poly(meth)acrylates or alginates), otherwise the solubility (if the compound is hydrophobic) may be exceeded, with the consequent formation of crystals and decreased absorption (Acosta 2009).

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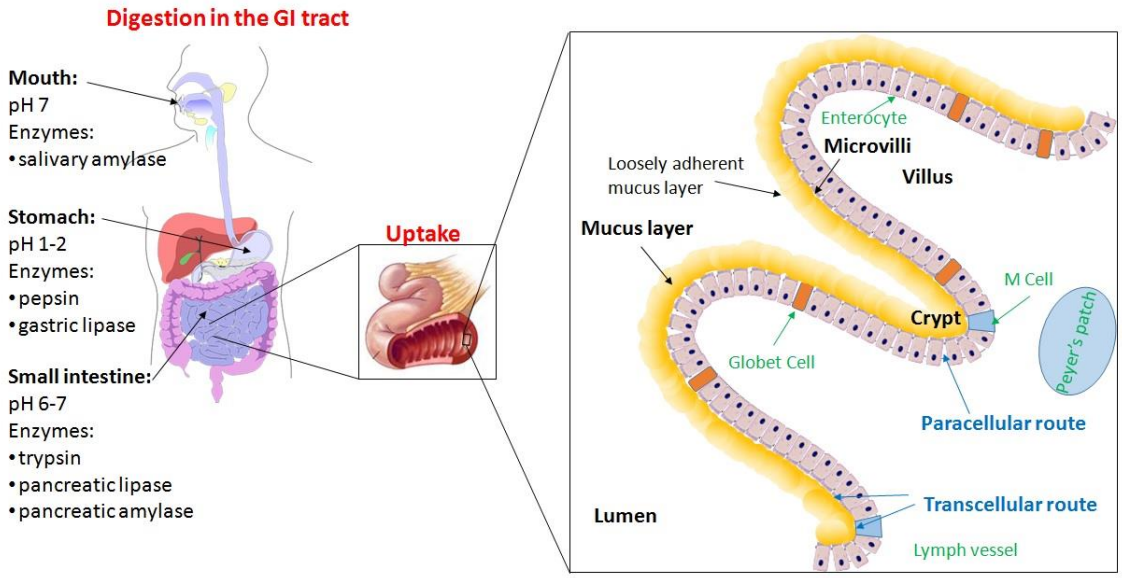

Figure 9.1. Schematic representation of the main GI barriers for bio-nanosystems digestion and absorption.

Bearing in mind how the process of digestion and absorption occurs, we will now introduce the advantages that bio-nanosystems may offer, as oral delivery systems of bioactive compounds. Food-grade ingredients, such as polysaccharides, proteins, lipids and low molecular weight surfactants are widely used in the fabrication of these nanosystems for oral delivery, being biodegradable and non-toxic. They protect the cargo from degradative GI environment and the subcellular size improves not only sensorial aspects, but also solubility and bioavailability. This improvement in bioavailability seems, in most cases, to be related to the direct uptake of the nanocarriers (Acosta 2009). This may be linked to the larger surface area-to-volume ratio and physicochemical interactions at the nanoscale (Cerqueira et al. 2014). These interactions include mucoadhesion and permeability enhancing properties, which potentially improve the absorption across intestinal epithelial cell membrane. All these attributes are related to a smaller size. Indeed, nanoparticles in the range of 100 nm can freely diffuse through intestinal mucus *ex vivo* as compared to larger particles (500 nm), which is consistent with the reported mucus pore size within the range of 200 nm (Bajka et al. 2015).

This can also be explained by an increased retention, time and degree of interaction between the nanocarriers and the mucus layer in the small intestine. In this sense, it is known that positively charged polysaccharides such as chitosan interact with negatively charged mucin and components in the intestinal epithelial membrane (Shukla et al. 2013). This suggests that electrostatic interactions can drive mucoadhesion, although physical entrapment by the mucus layer can also take place, as well as hydrophobic and van der Waals interactions, and polymer chain penetration (Ensign, Cone, and Hanes 2012). In fact, chitosan introduces hydrophilic groups on the surface of the nanocarriers, which promotes the translocation across cellular cytoplasm. However, if the electrostatic interactions between the positively charged surface of nanocarriers and negatively charged mucin are too strong, the nanocarriers will be entrapped in the mucus without permeating through the epithelial tissue (Hussain, Jaitley, and Florence 2001). This may be ameliorated by the adsorption of bile salts and fatty acids, which can impart negative charge to the surface of nanocarriers and enhance the transport across the mucus network (Macierzanka et al. 2011).

When bio-nanosystems are incorporated into food or beverage products, they should be stable in the food formulation. Although they can influence its appearance, texture, stability and flavor, they must avoid aggregation, undesirable release and loss of activity of the encapsulated compound in the food matrix during storage before consumption. Therefore, interactions within the food matrix before and after intake need further understanding to unravel the fate during digestion and absorption.

Summarizing, the factors to take into account when designing an oral delivery nanosystem are as follows: compatibility between nanosystem materials and bioactive compound, biocompatibility of the synthesis procedure (such as organic solvent-free methods) and minimal processing of sensitive substances (avoid heat or vigorous agitation). In addition, solubility of bioactive compound in the nanosystem, high loading capacity, preservation and

protection during storage and behavior within the GI tract (susceptibility to chemical or enzymatic hydrolysis) (McClements et al. 2009) are all important, especially when targeting absorption at specific locations.

9.3. In vivo and in vitro experiments for bioaccessibility and bioavailability evaluation

The process of evaluation of digestion and uptake of bio-nanosystems in food containing bioactive compounds starts with the modelling of *in vivo* characteristics by using *in vitro* methods. This is typically followed by *ex vivo*, *in situ*, and *in vivo* techniques for validation.

The simplest *in vitro* experiments involve the physico-chemical characterization of the nanosystems behavior in simulated GI fluids. There is a standardized static digestion model (Minekus et al. 2014) that allows direct comparison across different laboratories. This includes the appropriate average pH, ionic strength and enzyme activity to mimic the physiological conditions of the gastric and intestinal fluids in sequence. This may serve as a preliminary test of the behavior of nanosystems containing bioactive compounds within the GI tract, to screen colloidal and chemical stability, enzyme degradation, release of encapsulated compounds and bioaccessibility. However, static models do not reproduce the dynamic aspects of GI physiology, such as progressive acidification and emptying from the stomach, gradual secretion of enzymes or mixing profiles or peristaltic contractions, and since these can affect the kinetics of nutrient bioaccessibility, there is the need to develop a standardized dynamic model. There is currently a model that reproduces pH gradient in the stomach, stomach emptying or gradual release of gastric enzymes, referred to as semi-dynamic method (Mulet-Cabero et al. 2017), since the dynamic aspects of the small intestine are not developed yet. Further, more sophisticated *in vitro* dynamic gastric simulators have been developed (Figure 9.2) and further

details can be found elsewhere (Verhoeckx et al. 2015), but these models are too complicated and costly to run on a daily basis.

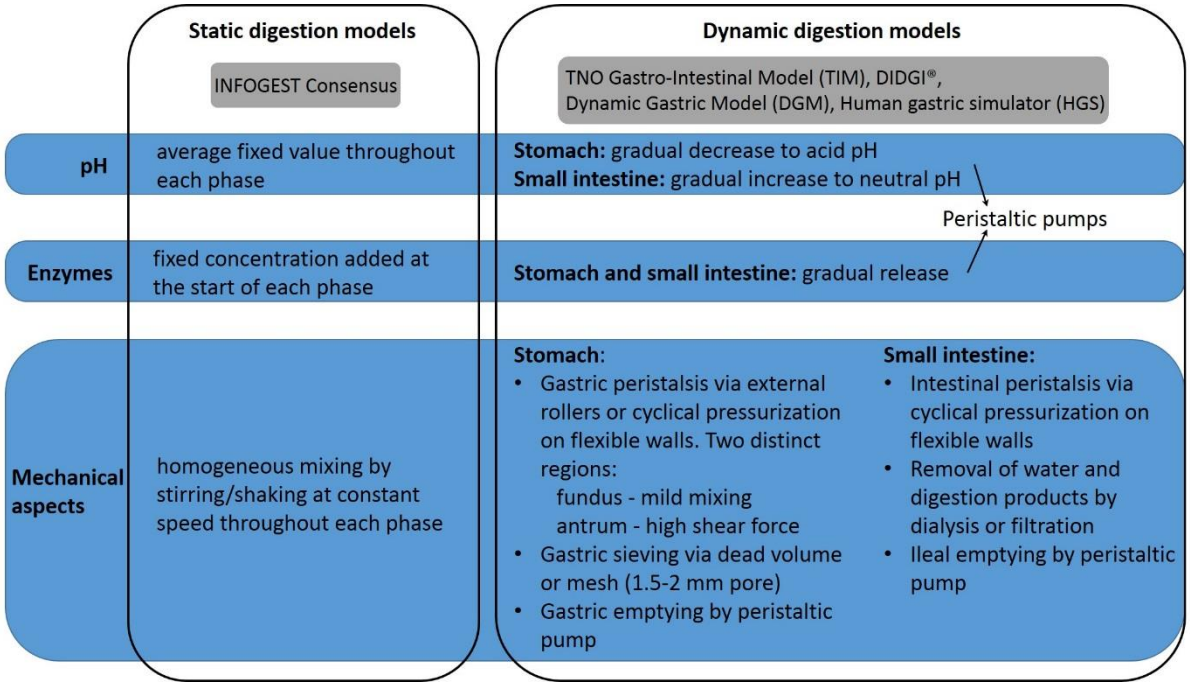

Figure 9.2. Summary of the main features of static and dynamic *in vitro* digestion models with emphasis on stomach and small intestine.

In order to test the transport of nanocarriers or the encapsulated compound across the intestinal epithelium, membrane systems are often used like dialysis bags or tubing containing the nanosystems (Sessa et al. 2014) and suspended in simulated GI fluids for a more accurate approach. However, these do not mimic the epithelial cell behavior. To achieve a more realistic model of the human gut epithelium, Caco-2 monolayer cell cultures or cocultures of Caco-2 can be grown in either a single cell culture well plate for uptake studies or in a membrane insert in a Transwell® system for transport studies (Gamboa and Leong 2013). Nevertheless, Caco-2 monolayer (human epithelial colorectal adenocarcinoma cell line) only reproduces

enterocytes and is not an accurate model for small intestinal tissue, but rather colonic tissue instead. Cocultures of Caco-2 and other cell lines may take into account aspects of the multicellular intestinal epithelium by including M cells (Caco-2 and RajiB or lymphocyte coculture) and mucus secreting goblet cells (Caco-2 and HT29 coculture). Good descriptions of the methods for these types of cell cultures are given elsewhere (Verhoeckx et al., 2015). A more advanced microdevice called gut-on-a-chip has upgraded the benefits of using Caco-2 cell cultures by implementing fluid flow and mechanical stress *via* vacuum microchambers alongside the microchannels that mimic the peristalsis in the GI tract (Kim et al. 2012). This increases the paracellular transport without compromising the integrity of the cell monolayer. In addition, human gut isolated microbial flora can be cultured on top of the Caco-2 monolayer. In all these techniques, sampling and composition analysis of the collected aliquots are required to measure the release/transport of bioactive compound from the nanocarriers.

Ex vivo studies usually involve working with segments of animal gut. This offers a better representation of the morphological and physiological features of the intestine, such as the presence of all the relevant cell types and architecture, and the presence of a mucus layer (Gunness et al. 2016). These can be used to follow the transport of nanocarriers or nutrients across the intestinal epithelium, permeability, absorption or interactions with the mucus layer (Norris and Sinko 1997). The simpler techniques are intestinal rings and intestinal segments, where these are isolated and immersed into highly oxygenated buffer containing the compound of interest. The tissues are viable for 1 to 2 hours depending whether the muscle layers are present or removed, respectively. The main disadvantage is that the exposure to luminal (apical) and serosal (basolateral) side is not made via individual compartments, therefore this procedure is mainly used to measure the accumulation of nanocarriers, in this case into the enterocytes rather than transport (Hillgren, Kato, and Borchardt 1995). The individualization of apical and basolateral compartments can be achieved with the everted sac model, whereby

a segment of intestine can be sutured at one end, nanosystems introduced and the open end also sutured, and immersed in physiological solution, such as Ringer (Trapani et al. 2010). Another alternative is opening the intestinal segment so that the tissue can be mounted in an Ussing chamber, where the tissue is set on a frame dividing two semi-chambers, one facing the apical side, where the nanosystems are loaded, and the other facing the basolateral side (Lundquist and Artursson 2016). The electrophysiological properties: transepithelial electrical resistance, potential difference between the two chambers and short-circuit current of the tissue are monitored throughout the experiment as indicators of tissue integrity and viability. Most of these intestinal tissue models make use of an animal source, rats, rabbits and pigs being the more common, due to the limited availability of healthy human intestinal tissue. Although pigs share more physiological and immunological similarities to human than rodents, the extrapolation of data to humans is complicated due to interspecies differences (Rowan et al. 1994), even the large inter-individual variability in humans make the interpretation of the results difficult in small studies. The use of human intestinal tissue in Ussing chambers has been discussed in a review by Lundquist and co-workers (Lundquist and Artursson 2016), who include a limited number of studies of nanoparticle transport across human intestinal tissue. Once more, sampling is required from basolateral and luminal sides to determine the concentration of the bioactive compound and assess uptake across epithelial layer.

The most common *in situ* technique is the intestinal loop model. This method requires the animal to be under anaesthesia during the procedure, in which a segment of the intestine is ligated to form a loop, the nanocarrier suspension injected into the loop and this returned to the body cavity for up to 2 hours. Then the animal is sacrificed and the loop or the entire intestine removed for analysis (Desai et al. 1996).

The most relevant information that can be obtained from *in vivo* studies includes compound release kinetics and biodistribution of the nanocarriers (Gamboa and Leong 2013). Non-

invasive imaging techniques are usually preferred, although information is limited. These focus on stomach, small intestine and the colon. Radiolabelling can enable monitoring and quantifying nanoparticles on their transit through the GI tract in real time. Nevertheless, it can be an invasive technique and involves radiation exposure. In addition, analysis of blood is performed to determine plasma level of the considered bioactive compound after oral delivery and organ analysis is carried out to quantify tissue concentration (Gamboa and Leong 2013).

9.4. Evaluation of bio-nanosystems containing bioactive compounds within the GI

Until 2014, most of the reviews on nanosystems for oral delivery and the behavior of these within the GI tract were largely focused on polymer-based micelles and lipid-based nanosystems such as nanoemulsions (Cerqueira et al. 2014) because of the scarce data on other nanosystems. The fact that most of the bioactive compounds such as fatty acids, carotenoids and tocopherols, are lipophilic, justifies the relatively larger amount of studies on their encapsulation in lipid-based nanosystems (Tamjidi et al. 2013). In addition, the presence of digestible lipids facilitates the absorption of these bioactive compounds in the small intestine.

In this section, information will be provided about the behavior of these and other nanosystems encapsulating bioactive compounds within the GI tract in models where enzymes are included. Data regarding monitoring the rate and extent on digestion, and therefore the release and bioaccessibility of bioactive compounds as indicative of potential bioavailability will be also reviewed in critical manner. In addition, we also highlight *in vivo* studies of uptake and bioavailability of bioactive compounds encapsulated in bio-based nanocarriers. Bioactive compound loading, encapsulation efficiency, shelf life stability, as well as sensory properties or consumer acceptability are not part of the main discussion within the current chapter.

9.4.1. Nanohydrogels

Nanohydrogels are defined as nanosized hydrogel particles formed by physically or chemically cross-linked hydrophilic or amphiphilic polymer network (Shin, Kim, and Park 2015). The availability of the interior network for the incorporation of bioactive compounds can potentially increase uptake, absorption and bioavailability (Cerqueira et al. 2014). The water holding capacity and permeability are the key features of these nanosystems in addition to responding to environmental stimuli for triggered delivery system (Shin, Kim, and Park 2015).

Proteins and proteins-polysaccharides nanohydrogels have been developed. The main limitation of these systems under physiological conditions is that the labile bonds in the polymer backbone or in the cross-links can be hydrolyzed by enzymes. It has been reported that when comparing nanohydrogels comprising only protein or protein and polysaccharide, an external coating of polysaccharide, such as chitosan or alginate, offers better protection of the encapsulated bioactive compound under *in vitro* gastric conditions (Somchue et al. 2009). This is due to the slower rate of protein hydrolysis, greater particle stability (Bourbon et al. 2016), as well as prolonged release in the simulated intestinal phase (Somchue et al. 2009) .

9.4.2. Nanocapsules/nanoparticles

Nanocapsules or nanoparticles are formed by an external polymeric membrane and an internal part constituted by a liquid or polymeric matrix containing the bioactive compound. Three categories can be classified according to the material composing the nanoparticles: polysaccharide, protein or both.

9.4.2.1. Polysaccharide-bioactive compound nanoparticles

Polysaccharides are considered beneficial for improving the intestinal absorption of active ingredients, above all those that are water-soluble, but have low permeability in the small intestine (Hu et al. 2017). Therefore, the development of nanoparticles with this type of food ingredient can be beneficial.

Nanocomplexes formed by amylose and lipids were proposed as delivery vehicles for polyunsaturated fatty acids (PUFA) within the small intestine. *In vitro* digestion in simulated stomach showed high retention of conjugated linoleic acid, however a high rate of hydrolysis of amylase in simulated small intestine was observed with subsequent release of linolenic acid (Lalush et al. 2005). The high rate of hydrolysis of amylase might also potentially be an issue in the stomach if the pH is sufficiently high for the salivary amylase to remain active.

Water-soluble nanoparticles developed with low-molecular weight chitosan efficiently encapsulated lutein and improved the *in vitro* bioaccessibility, after simulated GI digestion and *in vivo* bioavailability in mice, according to higher concentration observed in plasma, liver and eyes, as compared with lutein in mixed micelles of lipid, bile and cholesterol as a control (Arunkumar, Prashanth, and Baskaran 2013). Nuclear magnetic resonance (NMR) experiments showed that weak bonds were formed between lutein and cross-linked chains of chitosan in the presence of water molecules.

The inclusion of cyclodextrins in hybrid poly/oligosaccharides nanoparticles has been shown to improve the capacity of these nanocarriers not only to load poorly soluble drugs and hydrophilic molecules, but also to enhance their transport across intestinal mucosal barrier of frogs in *ex vivo* experiments (Trapani et al. 2010). The authors attributed this to particular interactions of the nanoparticles with the surrounding epithelium due to intrinsic

physicochemical properties of the nanoparticles added by the cyclodextrins (smaller size, potential chelating capacity).

Electrostatic interactions between oppositely charged polysaccharides, such as chitosan and gum arabic, were used to form a strong polysaccharide matrix to fabricate nanoparticles containing other emulsifying agents (Tween 80 and egg yolk phospholipids) carrying curcumin (Tan et al. 2016). These novel nanoparticles allowed a delayed and more effective release of curcumin into the simulated small intestine phase by increasing the stability of curcumin and nanoparticles under simulated gastric conditions, as compared to emulsion without polysaccharide coating.

9.4.2.2. Protein-bioactive compound nanoparticles

Food proteins have extraordinary binding capacity to drugs or nutraceuticals via hydrophobic interaction, electrostatic attraction, hydrogen bonding, and van der Waals force. Thus, protein nanoparticles can be used to encapsulate nonpolar, polar, or charged compounds (Teng, Li, and Wang 2014). They also have high nutritional value as a source of essential amino acids (Hu et al. 2017).

Bovine β-lactoglobulin nanoparticles were developed by cross-linking with glutaraldehyde and reducing the cross-linker concentration by increasing the encapsulated curcumin that acts in turn as a physical cross-linker, due to the great protein binding affinity already mentioned. These nanoparticles show different curcumin release profile behavior under *in vitro* gastric conditions at either fed stated (gastric pH 5) or fasted state (gastric pH 2). Namely, nanoparticles where disintegrated in the fasted state and readily released the encapsulated curcumin, as opposed to the stability and controlled release displayed by nanoparticles in the fed state (Teng, Li, and Wang 2014). Despite the known resistance of native BLG to pepsin

digestion (Otte et al. 1997), which is largely active at pH 2. The authors attribute the behavior of nanoparticles in the fasted state to the susceptibility of intermolecular amide bonds produced by the glutaraldehyde to pepsin cleavage.

Casein micelles are also a potential nanodelivery system of bioactive compounds such as vitamin D. Reconstituted casein micelles loaded with vitamin D incorporated in low-fat milk and fat-free yoghurt led to high stability of vitamin D under storage conditions, as well as *in vivo* bioavailability, as shown in human clinical trials (Haham et al. 2012, Levinson et al. 2016). This offers a natural alternative to synthetic emulsifiers, such as Tween 80, used in commercial dietary supplements of vitamin D, and better palatability of the product as confirmed by sensory evaluations. The *in vivo* human bioavailability of vitamin D encapsulated in casein micelles incorporated in fat-free yoghurt is also as good as that of vitamin D dissolved within the fat of low-fat yoghurt (Cohen, Ish-Shalom, et al. 2017). A follow up study, under *in vitro* conditions, attributes these results to the protection of vitamin D conferred by the casein micelles under gastric conditions by means of protein-vitamin binding and curd formation, which allows an increase in vitamin retention as compared to free vitamin (Cohen, Levi, et al. 2017).

Complexation between native and pre-heated soy proteins with bioactive compounds like curcumin also leads to improved stability under *in vitro* GI conditions and bioaccessibility over the free bioactive compound. Pre-heated soy protein isolate (SPI) has been shown to bind to a larger extent to curcumin. However, protein hydrolysis during GI digestion induces nanoparticle aggregation to a larger extent in pre-heated protein nanoparticles that impairs bioaccessibility of the bioactive compound (Chen, Li, and Tang 2015). On the other hand, complexation with curcumin improved protein digestibility for both native and pre-heated SPI.

The performance of different food proteins in the release of nanoencapsulated compounds has been compared. A recent study used whey protein isolate (WPI) nanoparticles showing that

they were more resistant to *in vitro* pepsin digestion. This led to a very limited release of the incorporated β-carotene in the simulated stomach, as compared to sodium caseinate or SPI nanoparticles, followed by release after intestinal trypsin digestion (Yi et al. 2015). This was attributed to the lower digestibility of the native globular BLG, the predominant component in WPI, due to the stable conformation of the folded β-sheet-structure. All protein nanoparticles increased the cellular uptake of β-carotene in Caco-2 cells in relation to control (free β-carotene). However, the protective behavior of WPI in the stomach makes WPI nanoparticles a good candidate for a sustained release in the small intestine as the site of absorption.

9.4.2.3. Protein-polysaccharide-bioactive compound nanoparticles

In order to overcome the drawback of protein digestibility in the gastric phase, complexation with polysaccharide offers better stability and protection against pepsin hydrolysis.

Self-assembly through electrostatic interactions between proteins and polysaccharides allows the formation of nanoparticles such as those composed by a zein or SPI core coated with carboxymethyl chitosan (CMCS) to encapsulate compounds like vitamin D (Luo, Teng, and Wang 2012, Teng, Luo, and Wang 2013). The chitosan coating provides better controlled release of vitamin D under *in vitro* GI conditions because CMCS becomes insoluble and forms a gel-like barrier in the polymeric matrix at gastric pH (1.4–2.0) due to the protonation of the carboxylic group. Such feature allows CMCS to delay significantly the decomposition of the protein matrix, thus minimizing the release of the embedded compounds in the stomach and maximizing their availability for intestinal absorption. In addition, the authors observed that the complex nanoparticles provided stronger interaction with vitamin D through hydrogen bonding in comparison to the ones formed with single ingredients.

Nevertheless, the combination of hydrophilic polysaccharide and protein creates mostly noncovalent interactions, which are sensitive to environmental conditions. Accordingly, aggregation or phase separation might take place arising from the binding at high biocompound loading. Maillard conjugation (non-enzymatic glycosylation by means of covalent bonds) between proteins (either milk or plant origin) and polysaccharides has allowed the development of nanocomplexes or nanoparticles to encapsulate polyphenols, to overcome the precipitation of protein caused by the strong binding affinity with polyphenols (Hu et al. 2017). These nanoparticles have been shown to be similar or better at retaining bioactive compounds as compared to complexes between native proteins and bioactive compounds, to have better compound stabilization under GI conditions and more controlled release (Xue et al. 2014) also improving its *in vitro* bioaccessibility (Davidov-Pardo et al. 2015, Qiu et al. 2017). This is attributed to the greater steric hindrance provided by the polysaccharide molecules in the shell of the nanoparticles and less susceptibility to GI enzyme digestion of the glycosylated protein. In this sense, the polysaccharide coating may also improve the controlled release of the encapsulated compound as compared to the protein coating (Chen, Ou, and Tang 2016).

Cross-linking of peptide-polysaccharide nanoparticles with genipin is another alternative to improve stability under simulated GI conditions and sustained release (Hu et al. 2014). The advantages of this approach are not only the use of a natural and non-toxic cross-linker, but also the higher stability than chitosan-based nanoparticles in biological fluids.

9.4.3. Lipid-based nanosystems

9.4.3.1. Nanoemulsions

In general, nanoemulsions enhance lipid digestibility as compared to conventional emulsions due to the increased initial interfacial area exposed to lipase action, therefore a greater release of the encapsulated compound would be expected. Nevertheless, the rate and extent of lipid digestion also depends on other initial parameters, such as the emulsifier type, oil type and oil content used and stability under GI conditions.

Salvia-Trujillo *et al.* showed under *in vitro* conditions that the rate and extent of lipid digestion increased with decreased initial droplet size of nanoemulsions, and therefore increased the bioaccessibility of β-carotene incorporated in the oil-phase (Salvia-Trujillo et al. 2013). In another study, the *in vivo* GI absorption of eicosapentaenoic and docosahexaenoic acids rich fish oils from nanoemulsion and conventional emulsion formulation was evaluated using a single pass perfusion rat model (Dey et al. 2012). The lipid absorption in the small intestine of the rats was increased by using a nanoemulsion formulation.

The emulsifier type can also have an impact on the interfacial stability upon digestion and permeability across the intestinal barrier. This will depend on the interactions with GI components and surface activity. Recent work reported on the effect of the surface charge of nanoemulsions on their physicochemical stability, digestion and release of encapsulated curcumin under *in vitro* GI conditions using a sophisticated human gastric simulator (Pinheiro et al. 2013). The authors showed that the cationic emulsifier induced greater destabilisation of the nanoemulsions as compared to non-ionic or anionic emulsifiers, leading to larger droplet size, and hence poorer curcumin bioaccessibility. This was attributed to alterations in the bile and lipase adsorption. Li *et al.* showed recently that a protein-stabilized nanoemulsion is as good as protein complexation to improve stability under *in vitro* GI conditions and permeability

of curcumin in Caco-2 cell monolayer model (Li et al. 2015). The proteins used were dairy proteins (BLG and WPI), which show resistance to pepsin digestion, but are more susceptible to trypsin digestion, which is necessary for the release of the encapsulated compound. Another study tested the capability of nanoemulsions, stabilized mainly by soy lecithin or glycerol monooleate combined with either Tween 20 or sugar esters, to encapsulate resveratrol and proved its stability and excellent antioxidant activity (> 80 %) in CaCo-2 cells and by chemical assays under *in vitro* GI conditions (Sessa et al. 2011). This high antioxidant activity was related to better entrapment of resveratrol in the lipid phase due to the formation of reversed micelles of the more hydrophobic emulsifier within the lipid droplets stabilized by the more hydrophilic emulsifiers. This work suggests that nanoemulsions may improve the uptake of antioxidant compounds in their active form through the intestinal walls. In subsequent studies, the lecithin-based formulations of these nanoemulsions with encapsulated resveratrol were shown to enhance the transport of the antioxidant through CaCo-2 cell monolayers in shorter times than those required for their metabolization (Sessa et al. 2014). This was attributed to the more similar composition of the interfacial layers in lecithin-based nanoemulsions to that of the phospholipid bilayer structure of the cellular membrane. Modified starches can also be efficient stabilizers of nanoemulsions that improved the bioaccessibility of β-carotene under *in vitro* GI conditions as compared to β-carotene dispersed in bulk oil (Liang et al. 2013). In addition, decreasing the dispersed molecular density of the modified starch significantly enhances the bioaccessibility of β-carotene, which might be related to the thickness of the modified starch layer stabilizing the oil droplets.

The effect of the oil type has also been studied in the *in vitro* intestinal digestion of nanoemulsions and release of encapsulated curcumin (Ahmed et al. 2012). Specifically, the length of triacylglycerol chains (long: LCT, medium: MCT and short: SCT) had a marked effect on the rate and extend of release of free fatty acids and bioaccessibility of curcumin, the

latter estimated as the curcumin incorporated into the micellar phase after digestion. Specifically, LCT nanoemulsions had a slower rate and lower extent of lipid digestion, due to the inhibitory effect on pancreatic lipase caused by the interfacial saturation with the more water-insoluble long chain fatty acids. Following the trend of nanoemulsion digestibility one could also expect the lowest curcumin bioaccessibility in LCT nanoemulsions. However, SCT nanoemulsions showed the lowest curcumin bioaccessibility, despite the greater curcumin loading capacity, due to the high water-solubility of short chain fatty acids, which limits the capability for micelle formation. Similar trends were found for the rate and extent of lipid digestion of nanoemulsions and bioaccessbility of encapsulated vitamin D with varying oil type (MCT vs. LCT) (Ozturk et al. 2015). The LCT were found to improve the bioaccessibility of vitamin D due to better solubilization by long chain free fatty acids in mixed micelles.

The content of oil in the nanoemulsion formulations also plays a role in determining the rate of lipid digestibility, and hence bioaccessibility of encapsulated bioactive compounds under *in vitro* conditions. Xia *et al.* showed that nanoemulsions with lower fat content (4 %) were digested at a faster rate and to a larger extent and the bioaccessibility of the encapsulated β-carotene was accordingly higher than that from nanoemulsions with higher fat content (20 %) (Xia, McClements, and Xiao 2017).

9.4.3.2. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC)

Lipidic nanoparticles can be produced from nanoemulsions by using a lipid phase that can fully (SLN) or partially (NLC) crystallize at room and body temperature. Solid crystalline phase is present in both, thus the effect of physical state of lipid can be crucial on its digestion and release of encapsulated compounds.

SLN are formed by lipid droplets fully crystallized and have highly-ordered crystalline structure. Some studies have shown the high efficiency of SLN as compared to non-encapsulated compounds to stabilize and provide sustained and prolonged release under simulated GI conditions (Righeschi et al. 2016) and improve the oral bioavailability in animal studies (Pandita et al. 2014, Ramalingam, Yoo, and Ko 2016). One could hypothesize that the solid matrix of SLN will provide better stability to the encapsulated hydrophobic compounds against oxidation and display a slower rate of lipid digestion, and hence more controlled release of encapsulated bioactive compounds. However, a recent study showed that lipophilic molecules that are located within the lipid phase in emulsions are expelled from the core of the lipid droplet to the aqueous phase upon crystallization of the lipid phase in SLN, and thus their chemical stability is greatly compromised (Berton-Carabin, Coupland, and Elias 2013). The highly ordered structure formed by fat crystals leaves less space to accommodate the encapsulated compound (Tamjidi et al. 2013). In addition, the fat crystals present in SLN promote partial coalescence, leading to a poorer physical stability as compared to liquid lipid nanoparticles (Qian et al. 2013). Hence, this may affect subsequent stability and degradation under GI conditions. It has been recently shown by means of *in vivo* live imaging that SLN are efficiently digested in the mouse intestine, limiting the subsequent absorption of intact nanoparticles over the intestinal wall (Hu et al. 2016). This was also confirmed from results of *in situ* perfusion studies and transmembrane permeation across CaCo-2 cell monolayers.

NLC were developed to overcome the potential limitations related to SLN, as explained above, in addition to providing higher loading capacity and slow release (Yang, Liu, and Liu 2017, Shin, Kim, and Park 2015). NLC are formed from lipid droplets that are partially crystallized and have a less-ordered crystalline structure. NLC are a modification of SLN in such a way that the liquid lipid phase is located in the core of the solid lipid, therefore the bioactive compound is better dissolved in the liquid core and simultaneously encapsulated by the solid

shell. An *in vivo* study using bioluminescence imaging proved the prolonged retention of NLC stabilized with lipophilic emulsifiers in the rat abdominal region (Chen et al. 2010). Fang and co-workers compared the oral bioavailability of curcumin in suspension or encapsulated in NLC in rats after gastric administration and observed a significant increase in peak plasma concentration, which was also reached in shorter time, as well as an increase in area under the curve and tissue concentrations of curcumin when administered in the NLC (Fang et al. 2012). Similarly, cationic NLC increased the tissue concentration of quercetin after oral administration in mice as compared to quercetin suspension (Liu et al. 2014). This is hardly surprising given the limited solubility of curcumin in water. Most of the studies so far tested the *in vitro* digestion of NLC and release of encapsulated substances in NLC prepared with one type of liquid lipid and solid lipid at a fixed ratio. These studies showed the stability of NLC under GI conditions and increased solubility and release of encapsulated compounds in the simulated intestinal medium (Aditya et al. 2013, Park et al. 2017). A very recent study showed the effect of incorporating two different types of liquid lipids (MCT and LCT) at different ratios, in combination with one type of solid lipid in the NLC formulation on the rate of lipid digestibility and release of encapsulated curcumin (Yang, Liu, and Liu 2017). The authors showed that the rate of lipid digestion and release of curcumin from NLC increased with increasing the ratio of MCT:LCT in the nanosystem formulation. This was due to the preference of the lipase to selectively digest the MCT in the lipid mixture, which as explained above are faster hydrolysed as compared to LCT. The curcumin seems to better dissolved in the MCT phase, explaining the similar trend in the curcumin release profile as for the free fatty acid release.

9.4.3.3. Nanoliposomes

Nanoliposomes consist of a vesicle made of a phospholipid bilayer enclosing a small volume of aqueous liquid and can contain both water-soluble and lipid-soluble compounds.

Nanoliposomes were reported to efficiently encapsulate curcumin increasing its absorption in the GI tract and bioavailability in rats after oral administration, as compared to non-encapsulated curcumin or the mixture of curcumin and lecithin (Takahashi et al. 2009). Liposomes are also promising delivery systems of substances like carotenoids, which may provide improved bioaccessibility, as compared to nanoemulsions delivery systems, due to the solubilization capacity of the digested products that contains greater absolute amount of mixed micelles and lipid bilayers (Tan et al. 2014). However, leakage and fast release of the encapsulated bioactive compound are still a major disadvantage of liposomes as delivery systems (Tamjidi et al. 2013), therefore other solutions have been proposed to enhance their nanocarrier performance.

Nanoliposomes that were coated by chitosan showed an increased mucoadhesion in comparison with bare nanoliposomes prepared by the same method (Shin et al. 2013). WPI-coated liposomes containing quercetin in a dairy drink showed better stability, in terms of particle size and lower release of free fatty acids, under simulated gastric conditions than uncoated liposomes. This was attributed to the reduced semi-permeability of the membrane by the protein coating, which hindered osmotic effects affecting the particle size of the liposomes in the dairy drink matrix. Next, the free fatty acid release profile was greater for coated liposomes under simulated intestinal conditions, which may be linked to an improved release of the encapsulated compound (Frenzel et al. 2015).

9.4.4. Nanolaminated systems

Nanocomposites, and nanolaminated coating with the layer-by-layer (LbL) deposition can be designed so that its stability and properties change as a response to changes in environmental/physiological parameters (e.g., pH, temperature and ionic strength) (McClements 2010).

Oil-in-water emulsions nanolaminated with oppositely charged proteins (β-lactoglobulin and lactoferrin) at neutral pH values have been shown to be stable under storage conditions. However, they all (mono-, bi- or multi-layered) aggregated under *in vitro* GI conditions and did not have an impact in the digestion of triglycerides since protein was readily digested. The decreased bioaccessibility of β-carotene was attributed to binding with lactoferrin (Tokle, Mao, and McClements 2013).

A study reporting the *in vitro* digestibility of hybrid nanoparticles, fabricated with the LbL deposition of lactoferrin and bovine serum albumin onto liposomes, showed greater stability and more controlled and sustained release of the encapsulated compound from mono- and double-layered coated liposomes, as compared to bare liposomes under simulated intestinal conditions (Liu et al. 2017). The release under simulated conditions was similar and limited from all studied nanoliposomes. The nanolaminated coating in liposomes overcomes the drawbacks associated with the dynamic nature of the lipid bilayer, such as rapid fusion and aggregation between liposomes and substrates when incorporated in food matrices.

LbL deposition of polysaccharides (chitosan and alginate) onto liposomes also showed similar trend of controlled lipid digestibility and release of encapsulated compound as compared to bare nanoliposomes (Liu et al. 2013). Complexation of polysaccharides can reduce the porosity and decrease the leakage of the encapsulated ingredients more effectively than either alone.

Future trends and perspectives

Further research should assess the safety and potential side effects, since the biological fate of biomaterials is altered due to the smaller size in nanosystems. Indeed, although these nanosystems are made with food-grade ingredients, they might cause undesired effects such as transporting or depositing active ingredients or excipients in tissue that they are not supposed to, or enhancing the absorption of substances that they are not meant to transport, but that are present in the food matrix. In addition, optimization of bioavailability of bioactive compounds encapsulated in these bio-nanosystems could be achieved by use of reverse engineering approach. The use of more realistic and standardized *in vitro* digestion models will allow easier comparison across research laboratories for bioaccessibility results. One recent research evidenced the differences in particle size and bioaccessibility of β-carotene from SLN after applying static or dynamic *in vitro* digestion models (Gomes et al. 2017). The more realistic dynamic digestion model resulted in lower bioaccessibility of the encapsulated β-carotene, however, provided more reliable data related to release of free fatty acids, as compared to the simpler static digestion model.

The confluence of pharmaceutical, nutrition, and colloid sciences with food engineering will be the key to unlock the full potential of bio-nanosystems containing bioactive compounds in food applications. Future trends in nano-delivery systems should concentrate on the interactions between food components and nanoformulations in food systems, as well as their fate within the GI tract, since there is very scarce work of digestion and uptake of nanosystems incorporated in food products.

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