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# Towards the understanding of the behavior of bio-based nanostructures during *in vitro* digestion

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The encapsulation of bioactive compounds in bio-based nanostructures is considered a hot topic in food technology, due to their huge potential in protecting the valuable bioactive compounds and providing new functionalities (e.g. increase of bioavailability). However, there are still some challenges that must be overcome before this technology can be entirely embraced by food industry, including the optimization of nanostructures' formulations to increase stability and bioactive compounds' bioavailability and the risk assessment of their use in food. Therefore, in recent years, efforts are being directed to the evaluation of the *in vitro* behavior of nanostructures during digestion/absorption. This evaluation can be challenging, however, there are opportunities to take advantage from the lessons learned from pharmaceutical industry and of the considerable progress in the development of more realistic *in vitro* models and *in situ* analysis techniques to more accurately predict the behavior of bio-based nanostructures once ingested.

## Addresses

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## Introduction

The increase in consumers' awareness of the impact that food has on health has spurred the development of functional foods, that is, food containing bioactive compounds (e.g. carotenoids, vitamins, fatty acids, polyphenols, bioactive peptides, phytosterols, fibers and essential minerals) dispersed within their matrix. However, most bioactive compounds present low water solubility, poor

bioavailability, sensitivity to deterioration during processing, shelf-life and digestion, and/or unpleasant sensory attributes, and therefore cannot be added directly into foods [1<sup>\*</sup>]. Delivery systems at nano-scale have attracted considerable interest worldwide over the past decade, due to their potential to enhance the bioactive compounds' bioavailability (i.e. amount of ingested bioactive compound that is absorbed and available for physiological functions) and stability, with minimal adverse sensory effects. However, alongside their great potential, nanostructures for food applications are also facing some concerns about possible toxicity. Only through the understanding of the digestive fate of nanostructures/bioactive compounds it is possible to improve their performance and to conclude about nanostructures' safety. The present review highlights the latest (past 2–3 years) important advances in the development of bio-based delivery systems at nanoscale and in unraveling their behavior within the gastrointestinal (GI) tract, including an insight into the challenges associated with this *in vitro* evaluation and potential future directions (e.g. development and use of more sophisticated dynamic *in vitro* digestion models).

## Bio-based nanostructures for encapsulation of bioactive compounds

By contrast to pharmaceutical industry, nanostructures for food applications must be entirely produced with food grade ingredients (e.g. lipids, proteins, polysaccharides, surfactants) and through approved processing operations (e.g. mixing, homogenizing thermal processing) [2]. Because of this reason, there is an increasing interest in founding new natural ingredients to produce bio-based nanostructures with good properties to incorporate in food products without interfering with their sensory characteristics.

There have been major advances in the design and production of bio-based nanostructures to encapsulate bioactive compounds for food applications. Till date, different bio-based nanostructures have been described in the available literature, including nanoemulsions, liposomes, nanostructured lipid carriers, polymeric nanoparticles, protein–polysaccharide complexes and nanohydrogels. These bio-based nanostructures present different sizes, structures, compositions and physico-chemical properties and can offer numerous functionalities. Also, they typically present as principal advantages

biodegradability, low toxicity, high loading capacity, improvement of oral absorption and controlled/sustainable release [3]. Also, bio-based nanostructures can be especially formulated to survive passage through different regions of the GI tract and then release the bioactive compounds at a specific location, thus maximizing their potential health benefits [4]. Some recent developments on nanoencapsulation of bioactive compounds for food applications, with special emphasis to their outcomes, can be found in Table 1.

In the past years, there has been a tendency to design new bio-based nanostructures through the combination of existent nanomaterials (lipids and polymers), promoting a synergistic interaction between these nanomaterials. Furthermore, the development of new delivery systems, such as the combination of two (nano)structures (e.g. nanoparticles trapped within hydrogel beads [16]) could be a new approach for encapsulation of bioactive compounds with more stability, protection and efficient control release in food products.

Table 1

## Recent advances in the development of bio-based nanostructures for encapsulation of bioactive compounds for food applications

Nano-delivery system	Bioactive compound	Nanostructure materials	Outcomes	Reference
Nanoemulsions	Astaxanthin Lycopene	Linseed oil Tween 20	<ul style="list-style-type: none"> <li>✓ Higher stability to storage and environment conditions for emulsions with size lower than 200 nm</li> <li>✓ Incorporation of combined antioxidants increase the oxidative stability</li> <li>✓ Higher bioaccessibility of carotenoids encapsulated in nanoemulsions</li> </ul>	[5]
	Pterostilbene	Olive oil Flaxseed oil Tween 20	<ul style="list-style-type: none"> <li>✓ Higher percentage of FFA released for olive oil</li> <li>✓ Similar bioaccessibility of pterostilbene for olive oil and flaxseed oil nanoemulsions</li> <li>✓ Potential enhancement of the permeability of the tight junctions for olive oil nanoemulsions</li> </ul>	[6]
Liposomes	Fish oil	Soy lecithin Sunflower oil	<ul style="list-style-type: none"> <li>✓ Encapsulation efficiency ≈92%</li> <li>✓ Good stability</li> <li>✓ Incorporation in yogurt did not affect its sensory quality and protected the fish oil</li> </ul>	[7]
	Catechin Epigallocatechin gallate (EGCG)	Soy lecithin	<ul style="list-style-type: none"> <li>✓ High retention of bioactive compounds in the cheese structure</li> <li>✓ Increase of the antioxidant activity and total phenolic content without affecting the cheese characteristics</li> <li>✓ Good protection of bioactive compounds</li> </ul>	[8]
Nanostructured lipid carriers	Terpene	Beeswax MCT Alkylpolyglucoside	<ul style="list-style-type: none"> <li>✓ Good stability</li> <li>✓ High loading capacity</li> <li>✓ Biphasic release: burst release and sustained release</li> <li>✓ Profile release dependent on chemical structure of terpenes</li> </ul>	[9]
	Vitamin D3	Oleic acid Glycerol monostearate Tween 80	<ul style="list-style-type: none"> <li>✓ High encapsulation efficiency, that is not affected by differences in pH</li> <li>✓ High stability in acidic conditions, but instable in alkaline conditions</li> <li>✓ Good storage stability</li> <li>✓ Capability to control release</li> </ul>	[10]
Polymeric nanoparticles	Curcumin	Chitosan Gum arabic Tween 80 Egg yolk phospholipid	<ul style="list-style-type: none"> <li>✓ High encapsulation efficiency and loading capacity</li> <li>✓ Good physical stability</li> <li>✓ Improvement of antioxidant activity</li> <li>✓ Enhancement of delayed release in GI tract</li> </ul>	[11]
	CoQ <sub>10</sub>	Octenyl succinic anhydride modified starch (OSA-ST) Rice bran oil	<ul style="list-style-type: none"> <li>✓ Good thermal and pH stability</li> <li>✓ Encapsulation efficiency ≈98.2%</li> <li>✓ Suitable for use in beverages, fruit juices and baked goods</li> </ul>	[12]

**Table 1** (Continued)

Nano-delivery system	Bioactive compound	Nanostructure materials	Outcomes	Reference
Nanohydrogels	Curcumin Caffeine	Lactoferrin Glycomacropeptide	<ul style="list-style-type: none"> <li>✓ Capability to incorporate hydrophilic and lipophilic compounds</li> <li>✓ High encapsulation efficiency</li> <li>✓ Enhancement of bioactive compounds' antimicrobial activity</li> <li>✓ Bioactive compounds' release dependent on pH</li> </ul>	[13]
	$\beta$ -Carotene	WPI Alginate	<ul style="list-style-type: none"> <li>✓ Better chemical stability than nanoemulsions during storage and within GI tract</li> <li>✓ Lower bioaccessibility than nanoemulsions</li> <li>✓ Useful for delivering bioactive compounds in the large intestine</li> </ul>	[14]
Protein-polysaccharide complexes	Fish oil	Sodium caseinate Gum arabic	<ul style="list-style-type: none"> <li>✓ Encapsulation efficiency <math>\approx</math>78.9%</li> <li>✓ Successful incorporation within juice fruit</li> <li>✓ Juice fruit enriched with oil fish nanoencapsulated presented a bioaccessibility <math>\approx</math>47.4%</li> </ul>	[15]

The selection of the most appropriate bio-based nanostructure for a particular application requires an understanding of the bioactive compound properties and of the nature of the food matrix in which it will be incorporated, as well as on their behavior within the GI tract.

### Gastrointestinal fate of bio-based nanostructures/bioactive compounds

Bio-based nanostructures undergo a series of complex physicochemical and physiological processes as they pass through the different regions of the GI tract, before the release of the bioactive compound (Figure 1). Some of the key processes are (i) consecutive changes in the pH of the medium, which could alter nanostructures' electrical charge, and consequently their composition, structure and interactions; (ii) variations in the type and concentration of ions, which may impact the electrostatic interactions in the nanostructure through electrostatic screening or binding effects; (iii) presence of surface-active components (e.g. phospholipids and bile salts), which could lead to changes in nanostructures' interfacial composition; (iv) presence of enzymes able to digest components of the bio-based nanostructure, such as lipids (lipases), phospholipids (phospholipases), proteins (proteases), and starch (amylases); (v) temperature, which may cause changes in the physical state, molecular conformation or interactions of specific components, impacting nanostructures' digestibility; and (vi) flow/force profile which, besides mixing the various components, could lead to the breaking down of the delivery system's structure.

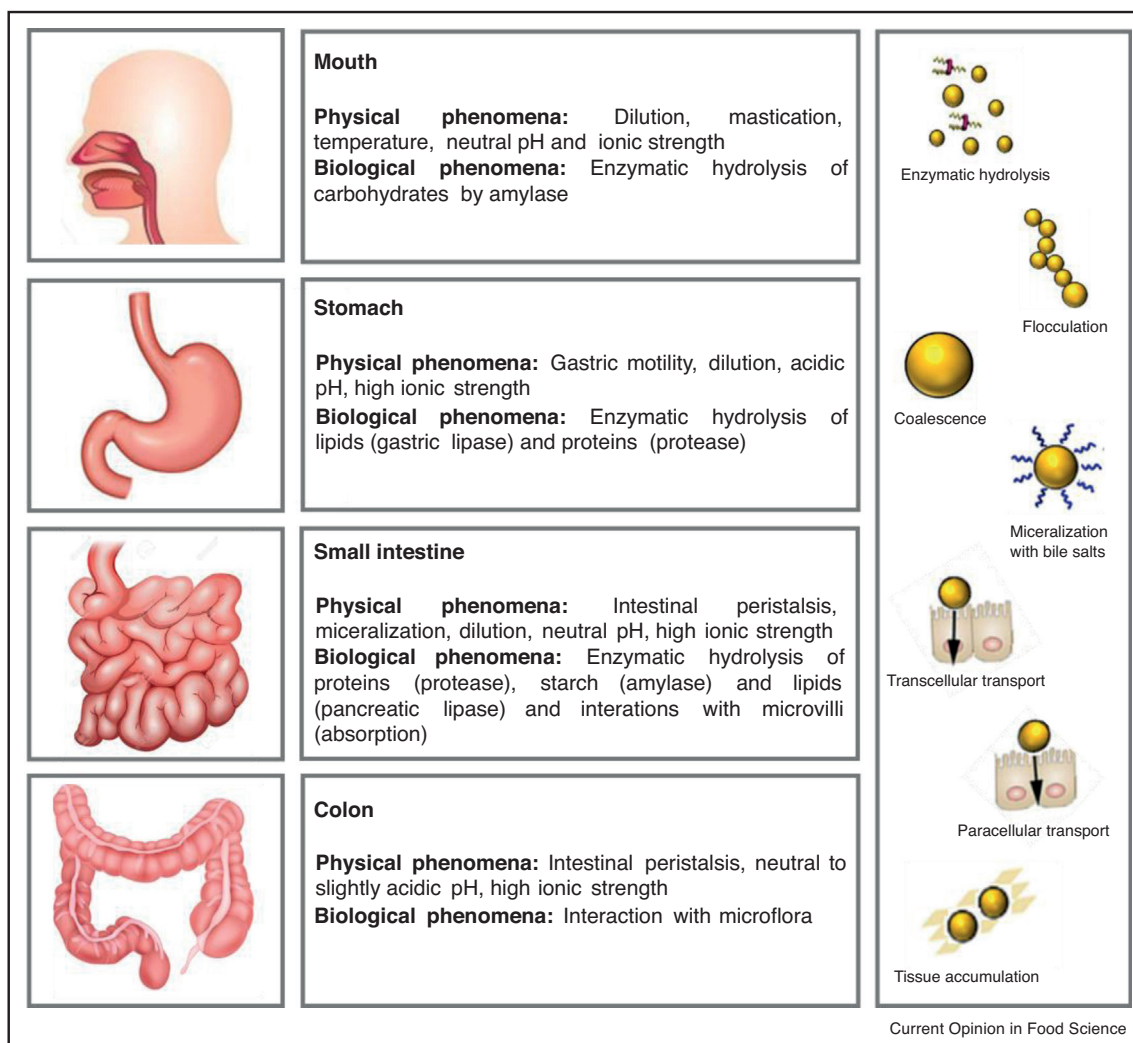
Different strategies have been used to control nanostructures' fate in the GI tract. For example, enzyme accessibility to a nanostructure may be controlled by creating physical barriers between the nanostructure and the surrounding aqueous phase containing the digestive

enzymes. It has been demonstrated that emulsions' behavior during digestion can be modulated by coating them with biopolymers [17], nanostructures [18] or conjugates [19].

Thus, the biological fate of bio-based nanostructures will be dependent not only on their initial physicochemical characteristics (e.g. composition, size, structure, interfacial properties and physical state), but also on the extension of the changes experienced as they pass within the GI tract. It is known that at nanoscale, the biological fate of the delivery systems and bioactive compounds incorporated within may be altered, influencing their absorption, distribution, metabolism and excretion, and consequently their potential toxicity [20]. However, in contrast to inorganic nanoparticles (e.g. gold, silver), the absorption of intact bio-based nanostructures into the systemic circulation is unlikely, due the changes after undergone gastric and intestinal digestion, being their accumulation in the organs, and consequent toxic effect, also improbable. In any case, the transformation and retention of bio-based nanostructures are related to their physicochemical properties, and therefore, the evaluation of their safety must be done in case-by-case basis.

Regarding the bioactive compounds, different mechanisms can be involved in their release from nanostructures (e.g. diffusion, swelling, erosion, fragmentation, dissolution, stimuli response) and, depending on the system and environmental conditions, a different mechanism may prevail [21]. Two main transport mechanisms can be involved in their passage across the intestinal epithelium: (1) between cells via tight junctions — paracellular route or (2) through intestinal membrane cells — transcellular route [22\*]. The bioactive compounds can undergo chemical degradation under harsh GI conditions or may be

Figure 1



Physiological and physicochemical changes that bio-based nanostructures can undergo as they pass through the GI tract.

metabolized by different enzymes during their passage from the GIT tract, to the epithelium cells, and then into the systemic circulation, leading to low bioavailability.

Understanding of the GI fate of bio-based nanostructures is therefore required to predict and increase their functionality (i.e. bioavailability of the bioactives compound) and to evaluate their potential toxicity.

#### Strategies for the enhancement of the bioactive compounds' bioavailability

Bioavailability is known to be dependent on the bioactive compounds' bioaccessibility, intestinal absorption and transformation in the GI tract [23]. The improvement of the bioavailability of a bioactive compound is essential to maintain their bioefficiency, that is, their health benefits. Several approaches have been used for the improvement of bioavailability of food bioactive compounds, most

of them translated from pharmaceutical drug research. However, in order to establish a successful strategy to enhance the bioavailability of the bioactive compounds it is important to know the main factors limiting their bioavailability.

The use of nanoscale delivery systems can be *per se* a strategy to increase the bioavailability of different bioactive compounds, once they could facilitate the entering of the bioactive compounds through biological barriers, as well as avoiding their metabolic modifications. In fact, nanostructures such as nanoemulsions or solid lipid nanoparticles have been shown to increase the bioavailability of different lipophilic bioactive compounds [24,25].

Also, there is a high potential to further increase the bioactive compounds' bioavailability through the improvement of their intestinal permeability, which can

be achieved by the incorporation of absorption enhancers in the delivery systems' formulation. Different food-grade compounds, including bile salts, surfactants, fatty acids, polymers and herbal bioactives have shown the ability to improve the intestinal absorption of bioactive compounds [26]. The mode of action of absorption enhancers can be either paracellular, by the opening of tight junctions, or transcellular by increasing the plasma membrane permeability, or a combination of both [27]. Other potential approach to improve the bioavailability of bioactive compounds is the inhibition of intestinal cell transporters (e.g. ATP binding cassette (ABC) transport proteins) once they can be involved in the efflux of bioactive compounds back into the intestinal lumen, reducing bioavailability [28].

Moreover, the co-ingestion of bioactive compounds with excipient foods is also a bioavailability enhancement strategy that has gained much attention in the recent years. In this case, the bioactive compound (that might be left in its natural source) is consumed with an excipient food specially formulated to control bioactive compounds' release, solubilization, transport, metabolism and absorption within the GI tract, boosting their bioavailability [29]. In fact, it has been suggested that delivery systems containing mixed colloidal particles (protein and lipid nanoparticles) may be designed to increase the chemical stability and bioaccessibility of lipophilic bioactive compounds [30].

### Challenges in tracking bio-based nanostructures digestion

There are still some challenges that must be overcome regarding the evaluation of bio-based nanostructures during digestion. For instance, digestion secretions (i.e. presence of enzymes, salts and bile salts), sample dilution (during digestion) and sample preparation (e.g. centrifugation) may influence the overall results obtained from the *in vitro* digestion (e.g. bioavailability determination or bio-based nanostructure tracking).

Centrifugation [31,32,33] and sample dilution [33] are often used as a pre-treatment, prior to sample analysis regarding spectrometry (i.e. spectrophotometry, spectrofluorimetry), chromatography (i.e. HPLC) and structural (i.e. DLS) analysis, respectively. Digestion samples may also be submitted to oxygen and light exposure, and temperature and pH variations, prior to analysis. For instance, *in vitro* digestion protocols often include an enzymatic deactivation step that may alter the structure of the assessed bio-based nanostructures or the bioactive compounds' activity. In fact, it is a common practice to submit digestion samples to low temperatures (i.e., through ice or liquid nitrogen) to slow the enzymatic digestion rates. Samples are also often submitted to high temperatures (i.e. 100 °C) to stop enzymes' activity.

Moreover, due to the complexity of the mixture after digestion, a separation and purification step are often required. Several methods can be used for separation and purification of bio-based nanostructures after digestion simulation, including asymmetrical flow field flow fractionation [34]. It is expected that in the next few years, new reliable methods for the detection and characterization of nanostructures under different conditions are established, improving the traceability of nanostructures within the human body.

Despite the fact that these procedures are in fact crucial to determine bioactive compounds' bioavailability and nanostructures behavior under digestion, they can also be considered as a bottleneck that needs to be addressed to minimize misconceptions and false results. *In situ* analysis can solve some of the challenges discussed above since it does not require sample pre-treatment procedures and gives more reliable information, in real time, regarding nanostructural changes during digestion so that correlations can be made toward bioactive compounds' bioavailability. Recently, a 'real-time quantitative method' to track the structural changes that occur in emulsion interface, during *in vitro* intestinal conditions has been developed and these changes were correlated with the release of free fatty acids. The authors used fluorescent resonant energy transfer (FRET) to evaluate, *in situ*, the emulsion digestion under simulated intestinal conditions [35].

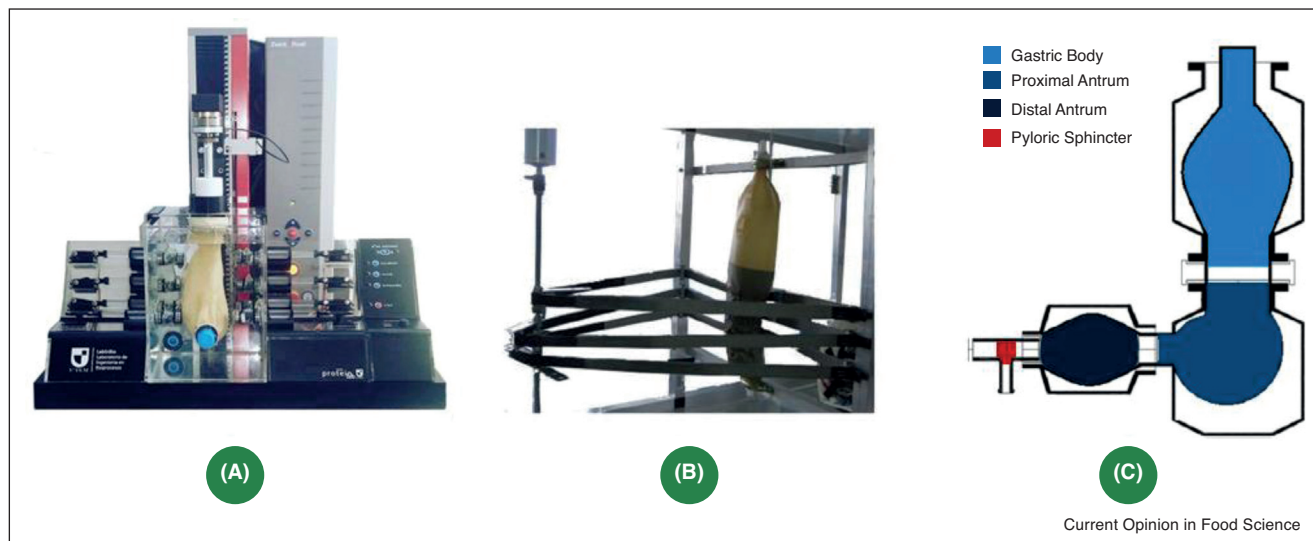
### Recent advances in the development of *in vitro* GI systems

*In vitro* GI models are currently used as the main tool to understand the behavior of bio-based nanostructures under GI conditions, minimizing the negative implications associated with *in vivo* studies (i.e., ethical, economical and technical constrains). However, these models are usually simple *in vitro* static models due to their lower cost and simplicity regarding protocol execution [36,37]. Only recently, dynamic *in vitro* GI models are being used to evaluate the behavior of bio-based nanostructures during digestion [38,39]. There is an ongoing awareness to develop more realistic *in vitro* GI models to study, not only food digestion, but potentially the behavior of nanostructures, reproducing the phenomena that occur during human digestion (e.g. gastric peristaltic movements) [40,41].

Recently an '*in vitro* mechanical gastric system' (IMGS— Figure 2a) using 3D printing technology has been developed. The authors used natural liquid latex and the model was coupled with four pairs of acrylic pistons, arranged on each side of 3D printed stomach to reproduce three contractions per minute. This system enables the study of important phenomena (i.e., propulsion, grinding and retropulsion) that occur during gastric digestion [40]. Other authors developed an '*in vitro* gastric device'



Figure 2



*In vitro* gastric models containing different mechanisms to simulate the human gastric peristaltic movements where (a) represents the IMGS, (b) represents the *in vitro* gastric device and (c) represents the TIMagc (reprinted from References [40\*,42,41], respectively, Copyright 2017, with permission from Elsevier (a and c) and Dissolution Technologies, Inc. (b)).

coupled with a peristaltic mechanical system consisting in a three belt apparatus (Figure 2b). The authors used latex to achieve the appropriate deformation characteristics of the human stomach, with an oval shape. The belts were strategically positioned at the top, middle and bottom of the stomach model, reproducing three contractions per minute [42]. Also, an ‘advanced gastric compartment’ (TIMagc — Figure 2c) has been developed to study food gastric digestion. The authors built three compartments simulating the gastric body, proximal and distal antrum and the model was coupled with a valve to mimic the pyloric sphincter. The stomach peristalsis was simulated by synchronously contracting inner membranes on each compartment, simulating the human stomach motility [41].

The development of novel gastric systems that mimic both the anatomy and peristaltic gastric movements that occur during *in vivo* digestion is showing to be a current trend. These models use extensive *in vivo* data to recreate the conditions of the human stomach regarding pH variation during bolus digestion, stomach motility and secretion composition.

Recent advances were also observed regarding cellular studies, as a means to study bioactive compounds permeability in the small intestine. A tissue-engineered model has been established, by co-culturing Caco-2 absorptive cells, HT29-MTX mucus-producing cells and Raji B lymphocytes and the presence of M-cells (derived from stem cells) has been accessed [43]. The authors concluded that the presence of M-cells improved the

bioactive compound transport efficiency, showing to be a more complete and reliable tool to perform permeability tests.

Despite the recent advantages in the field, there are still some bottlenecks that need to be addressed regarding the evaluation of bio-based nanostructures behavior under digestion. For instance, there is still a lack of agreement regarding the standardization of *in vitro* digestion protocols (i.e., both static and dynamic), despite the efforts of the COST action INFOGEST [34], so that inter-laboratory results can be compared and correlations can be established.

### Conclusions and future trends

This review focus on the recent advances on the development of bio-based nanostructures for encapsulation of bioactive compounds, with special emphasis to their behavior under GI digestion. Efforts are being directed to the development of the next-generation delivery systems, with maximum functionality. Some examples can include mixed nanoparticle systems, nanoclusters, Trojan-horse nanoparticles and environmentally responsive nanoparticles.

However, there is still a lack of knowledge on the behavior of the nanostructures/bioactive compounds encapsulated in the GI tract, in particular on their potential adverse effects on human health. In order to respond to this concern, more realistic *in vitro* digestive/absorption models and more advanced analytical methods need to be developed, in order to understand the fate of nanostruc-

tures within the GI tract, allowing the design of nanostructures with increased functionality and predicting the consequences of their intake. Even when using simple *in vitro* models, there is an evident need to develop standardized methods (e.g. for measure the bioavailability of bioactive compounds) that could allow a more accurate comparison of results between different laboratories. Finally, but not less important, a clear legislation and guidelines concerning their impact on human health and on environment must be assured. Although the regulatory framework on the use of nanostructures in foods is still in flux, it is expected an increase of control and regulation to assure the proper development and utilization of bio-based nanostructures in foods.

### Conflict of interest

The authors declare that there is no conflict of interest.

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