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Review

Emerging challenges in assessing bio-based nanosystems' behaviour under *in vitro* digestion focused on food applications – A critical view and future perspectives

Daniel Madalena [a], Jean-Michel Fernandes [a], Zita Avelar [a], Raquel F.S. Gonçalves [a], Óscar L. Ramos [b], António A. Vicente [a], Ana C. Pinheiro [a,*]

[a] CEB- Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal
[b] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, 4169-005 Porto, Portugal

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ABSTRACT

The current consumers' demand for high quality food products together with the growing awareness regarding the link between health and nutrition has led to the development of novel food products with added functionality. Such functionality can be modulated by adding bio-based nanosystems that can improve the bioaccessibility of bioactive compounds and facilitate nutrient absorption. However, these functional properties can be significantly affected by the adverse conditions (e.g., low pH, presence of enzymes, salts) of the gastrointestinal tract. As such, understanding the behaviour of such delivery systems under digestion conditions is of utmost importance and several analytical tools and *in vitro* digestion models have been used for this purpose. This review summarizes the latest updates on nanosystems' performance under *in vitro* digestion and provides critical insights related to important and complementary analytical tools (e.g., rheology, Raman spectroscopy, x-ray scattering) used to assess their performance throughout digestion. Furthermore, the most prominent and frequent challenges associated with such *in vitro* analyses are also described, together with the current trends regarding the development of *in vitro* digestion models and some considerations that should be undertaken for their validation. Efforts must be made towards developing reliable and standard *in vitro* digestion models that use sophisticated analytical techniques to further expand the knowledge regarding nanosystems' behaviour under *in vitro* digestion conditions.

1. Introduction

Many efforts have been driven towards the development of functional foods, i.e., food products that present additional properties beyond their nutritional value (Simões, Martins, et al., 2020). For this purpose, bioactive compounds have been used and incorporated into food products to confer e.g. anticarcinogenic, anti-inflammatory, antifungal, antioxidant, antimicrobial and antiviral properties, among others (Silva et al., 2019). Such compounds may present poor water solubility and susceptibility to the harsh digestion conditions, lowering their bioaccessibility and bioavailability (Mahalakshmi et al., 2020). To overcome this bottleneck, bio-based nanosized delivery systems (i.e., nanosystems) have been used to protect the integrity and functional properties of bioactive compounds (Ramos et al., 2019; Simões, Abrunhosa, et al., 2020). For this purpose, protein (Mahalakshmi et al., 2020), lipid (R. F. S. Gonçalves, Martins, Abrunhosa, Baixinho, et al., 2021) and polysaccharide (Zhongyuan Guo et al., 2020) matrices have been used as vehicles to deliver and protect bioactive compounds since they are generally recognized as safe (GRAS) materials that have the ability to form functional structures (i.e., nanoparticles, nanodroplets, nanofibrils, nanohydrogels and nanoemulsions) that release their content in response to external environmental stimuli (Wei et al., 2019). These materials should fulfil the requirements for the development of controlled delivery systems, namely, ensure the correct and timely release of bioactive compounds. Ideally, *in vivo* models should be used to assess the impact and toxicity of such systems and infer about their performance under digestion conditions. However, *in vivo* models are expensive, labour intensive, time consuming and present some ethical constrains (Berthelsen et al., 2019). Alternatively, *in vitro* digestion models have been widely used by pharmaceutical and food scientists in

* Corresponding author.
E-mail address: anapinheiro@deb.uminho.pt (A.C. Pinheiro).

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order to understand the behaviour and performance of nanosystems, evaluate their ability to release the bioactive compounds in the correct location (e.g., small intestine), and their toxicity using epithelial cellular lines (e.g., Caco-2 cells) (Wei et al., 2019).

Several review papers can be found in literature about this thematic, but the most recent and relevant ones address the use of *in vitro* digestion models regarding the performance of nanosystems (Berthelsen et al., 2019), the importance of bioaccessibility studies of bioactive compounds to assess their stability and release kinetics (Shahidi et al., 2019), the attempts to establish correlations between *in vitro* and *in vivo* models (Bohn et al., 2018) and physical and biochemical mechanisms of digestion (Macierzanka et al., 2019). Among the reviews available, very few critically discuss the different challenges associated with the use of conventional analytical techniques to assess the nanosystems'

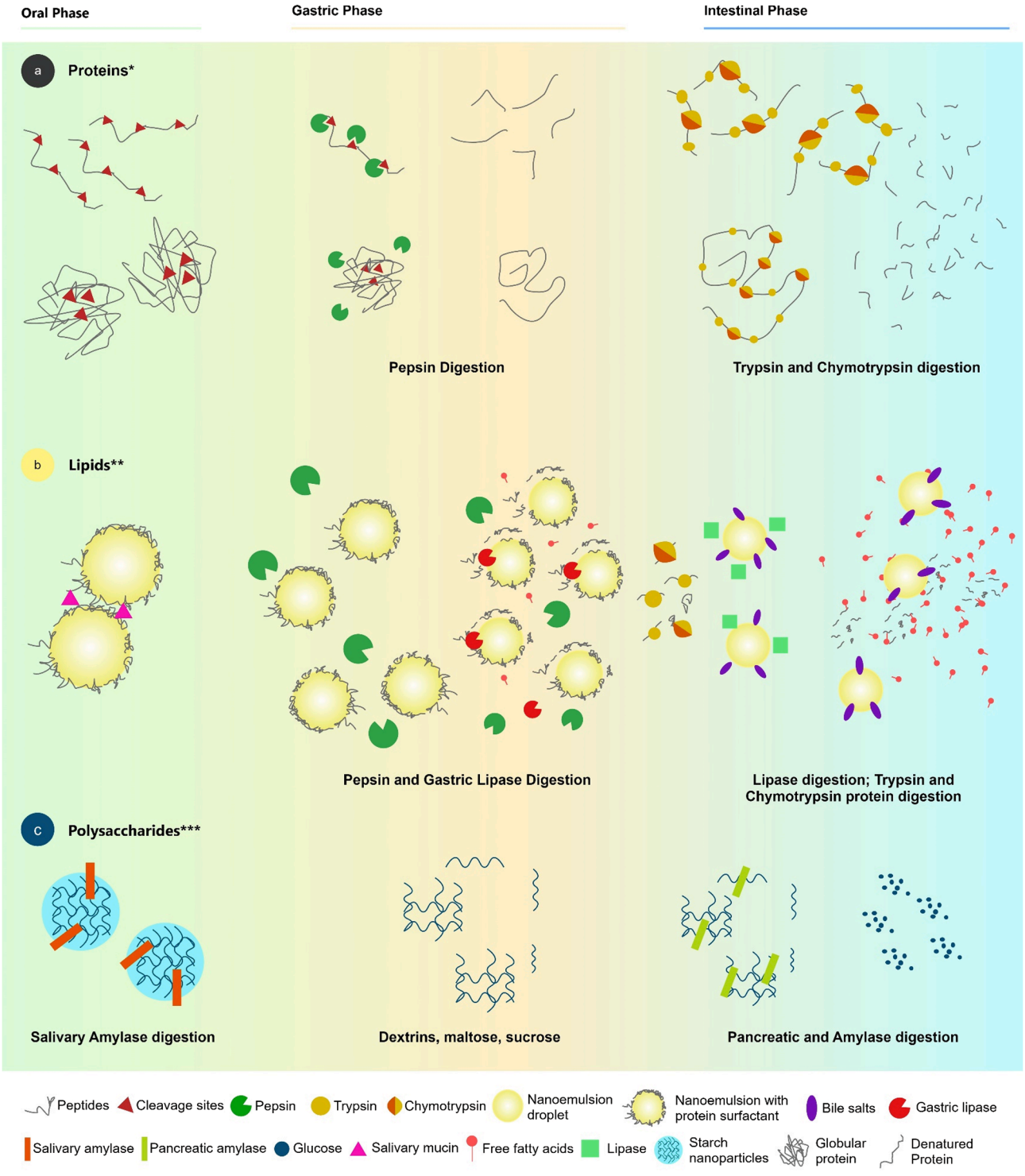


Fig. 1. Illustration of some important protein enzymatic interactions that occur during the gastric and intestinal phases of the digestion process. This figure is based on the work of Mackie & Macierzanka, (2010). *Protein digestion starts typically in the stomach; **Flocculation may occur from mucin interactions with adsorbed proteins; *** Polysaccharides are typically resistant to digestion in the upper GI tract and their digestion mainly occurs in the colon by the microflora with a few (though relevant) exceptions like such as starch, the which digestion process of which is depicted in this figure.

behaviour, specifically, under *in vitro* digestion as well as the challenges associated to the *in vitro* digestion models' validation. To fill these gaps, this review provides an up-to-date overview regarding the behaviour and characterization of bio-based nanosystems for food applications (i.e., protein, polysaccharide and lipid-based systems) under *in vitro* digestion, showing the current challenges of analytical techniques that can be used to assess their performance, and clearly presenting possible solutions to those challenges. Moreover, an update on the recent advances regarding the development of *in vitro* digestion models (i.e., static, semi-dynamic and dynamic models) that can be used to evaluate the nanosystems' behaviour under digestion, is presented in this review as well as their validation.

2. Behaviour of bio-based nanosystems under *in vitro* digestion conditions

Controlled delivery nanosystems must be capable of withstanding storage conditions as well as the harsh conditions of the upper gastrointestinal tract (GI) to be considered reliable delivery nanosystems. This means that they must resist the acidic pH of the stomach and the hydrolytic activity of enzymes (e.g., amylase, pepsin and lipase), to release their content in the appropriate location (Ahmad et al., 2019). If such requirements are met, nanosystems can be widely used to improve food quality and safety, shelf life stability, cost and nutritional value by the controlled delivery of several bioactive ingredients (e.g., polyphenols, vitamins, minerals, fatty acids, flavours, colours and preservatives) (Das et al., 2020).

This section highlights the behaviour and performance of protein-, lipid- and polysaccharide-based nanosystems under *in vitro* digestion conditions. An overview of the mechanisms that are responsible for the digestion behaviour of proteins, lipids and polysaccharides is depicted in Fig. 1, and these subjects are subsequently addressed in sections 2.1, 2.2 and 2.3 respectively.

3. Protein-based nanosystems

Proteins are very well known for their relevant functional properties such as emulsification, gelation, foaming and water binding capacity. These properties, together with their biodegradable nature, make them an ideal material for the development of nutraceutical bio-based delivery systems. Their structural versatility allows the production of films, particles, fibres, tubes and hydrogels for the delivery of both hydrophobic and hydrophilic compounds (Simões et al., 2017). Furthermore, proteins have high nutritional value and are GRAS materials (Simões, Martins, et al., 2020). Over the past few years, milk proteins, more specifically whey proteins, have been widely used as effective delivery vehicles for bioactive compounds. For instance, β-lactoglobulin, the major whey protein, is considered a suitable candidate to develop nanosystems due to its gastric stability and capability to bind hydrophobic constituents (Simões, Abrunhosa, et al., 2020; Simões, Martins, et al., 2020). However, the behaviour of protein nanoparticles highly depends on their electrical properties, i.e., their surface charge which in turn depends on the pH of the environment and the exposure of anionic and cationic groups. This way, their surface charge typically changes from negative to positive as the pH decreases and passes through the nanoparticles' isoelectric point where the surface charge is neutral and protein aggregation occurs (McClements, 2018a).

Protein nanohydrogels (or nanogels) have attracted much attention for the encapsulation of bioactive compounds due to their ability to swell in response to chemical (e.g., pH, solvent composition and ionic strength) or physical (e.g., temperature, electric or magnetic field, light and pressure) stimuli. Moreover, they are non-toxic and biodegradable materials that have a small size with a large inner network for multivalent bioconjugation (Araújo et al., 2020). Nanohydrogels consist in a three-dimensional network of hydrophilic polymeric molecules that can associate to each other through covalent or non-covalent (e.g., hydrogen bonds, van der Waals interactions and physical entanglements) interactions (Bourbon et al., 2019). Therefore, due to its stimuli-dependent response, hydrogels can e.g. release the entrapped bioactive compound into the media in the presence of alkaline pH, which makes them important delivery systems of bioactive food ingredients (Simões et al., 2017). For example, under gastric conditions, pH-sensitive hydrogels shrink, but under intestinal conditions they swell which results in the release of the entrapped bioactive compound through the increase of its mobility within the network matrix (Lv et al., 2018; Simões et al., 2017). This indicates that such delivery systems may be used to protect a bioactive compound from the harsh conditions in the human stomach (i.e., low pH and presence of enzymes) and release its content in the small intestine, improving nutrient solubility, bioaccessibility and consequently their bioavailability. G. Hu et al., (2021) used acylated ovalbumin nanohydrogels as protective carriers of curcumin under *in vitro* digestion conditions. These authors observed that ca. 23 % of curcumin was released during the digestion process which was significantly lower when compared to free curcumin control (ca. 87 %).

Similarly to nanohydrogels, protein nanoparticles can also be used as vehicles for bioactive compounds. As such, recent studies illustrate the potential of protein nanoparticles to be used as controlled delivery systems. For instance, Simões et al. (2020) investigated the *in vitro* digestion performance of β-lactoglobulin micro- and nanoparticles as protective carriers of riboflavin (vitamin B2). The authors showed that β-lactoglobulin nanoparticles with an initial size of 79.4 nm were able to retain 61.7 ± 4.1 % of riboflavin, allowing its release in the small intestine which improved its bioavailability. Moreover, the authors also observed an increase in particle size to ca. 4017.5 ± 497.6 nm and 600.8 ± 44.8 nm in the gastric and intestinal phase, as well as an increase in polydispersity to ca. 0.97 ± 0.05 and 0.66 ± 0.06, respectively, probably due to the low surface charge in the stomach (ca. 0.9 ± 0.5 mV) and consequently the absence of repulsive electrostatic forces that prevent particle aggregation. Casein nanoparticles can also be used to produce nanosystems to protect bioactive compounds during digestion. For instance, Du et al., (2022) assessed the performance of casein nanoparticles to encapsulate curcumin and evaluated their stability and curcumin release during *in vitro* digestion. The authors observed that the casein nanoparticles remain stable in terms of their size during the gastric phase of the digestion (i.e., size of ca. 107 nm) with a curcumin release of ca. 18.5 %. However, in the intestinal phase of the digestion, particle aggregation was observed, with particle sizes ranging from ca. 200 nm to 650 nm and a curcumin release of ca. 76.4 % which indicates that casein nanoparticles can significantly increase curcumin bioaccessibility.

Alternative sources of protein are also being used for the development of nanosystems. For example, soy protein isolate (SPI) nanoparticles were recently used as protective carriers for vitamin D3 during *in vitro* digestion. It has been shown that the incorporation of vitamin D3 in SPI nanoparticles treated with high pressure homogenization improves its release kinetics by ca. 18.8 % when compared with isolated vitamin D3. The authors observed that adding carboxymethyl chitosan, forming a protein-polysaccharide conjugate, significantly improved the performance of developed nanosystems by ca. 46.5 % and 37.2 % when compared with free vitamin D3 and SPI nanoparticles respectively (A. Zhang et al., 2020). In fact, the application of polysaccharides and lipids to improve protein-based nanosystems stability during *in vitro* digestion is common. For example, proteins can be conjugated with *k*-carrageenan (Lv et al., 2018), propylene glycol alginate (Wei et al., 2018), rhamnolipids (L. Dai et al., 2018), chitosan (W. Dai et al., 2020), among others, to improve bioactive compounds' stability and, consequently, bioaccessibility, during *in vitro* digestion (Bourbon et al., 2019).

Different protein digestion kinetics can be associated to different structural characteristics of protein nanosystems (depicted in Fig. 1a), which in turn are modulated by production processing techniques (e.g., heating, high-pressure, ultra-sound, among others) (Jin et al., 2021; Q.

Liang et al., 2021). For instance, protein digestibility can be modulated by thermal treatment since protein denaturation significantly changes its conformational state, making previously inaccessible chemical groups accessible to enzymatic interactions and thus proteolysis occurs (Rahaman et al., 2017). High pressure processing (i.e., pressure between 100 and 800 MPa) of proteins can also determine the fate of protein-based nanosystems within the GI tract. Studies show that high pressure treatments enhance protein digestibility by promoting partial (i.e., for pressures up to 450 MPa) protein denaturation and aggregation or complete (i.e., for pressures higher than 500 MPa) protein denaturation and partial aggregation (Kurpiewska et al., 2019; Xue et al., 2020). Ultrasonic treatments can also take a relevant role in improving protein digestibility. It has been reported that ultrasonic treatments may induce high pressures and shear forces that can promote significant changes to protein secondary and tertiary structures, particle size, charge and SH groups' exposure, which leads to an increase of protein susceptibility to pepsin and trypsin digestion (J. Li et al., 2018; Q. Liang et al., 2021). Proteins can also have a buffering effect during gastric digestion due to their ability to bind $H^{\pm}$ ions, specifically under acidic conditions and, consequently, change enzymatic activation and digestion kinetics. However, such effect is highly dependent on protein structure which in turn can be modulated by the aforementioned pre-processing (Mennah-Govela et al., 2020). Despite of the advances related to the production and optimization of protein-based nanosystems, further work is clearly required to better understand the behaviour of proteins within the GI tract as well as the functional compounds' release mechanisms at the nanoscale. Furthermore, tracking protein secondary structures during *in vitro* digestion is still a challenge. The interference of, e.g., enzymes, bile salts, among others, play a relevant role on the acquisition of circular dichroism spectra and, if properly obtained (i.e., without interferences) this information could lead to a better understanding of protein unfolding, hydrolysis and bioactive compounds' release kinetics during *in vitro* digestion. It is also important to mention that some proteins present some form of allergenicity, and this must be taken into consideration when choosing protein nanosystems for the oral delivery of bioactive compounds. For instance, protein allergenicity is partially associated to the resistance to the proteolytic effect of pepsin by some proteins (Mackie & Macierzanka, 2010). An example of this behaviour is β-Lg which is the main responsible for milk allergenicity (Kurpiewska et al., 2019). However, such allergenicity may be reduced by the previously discussed processing methods (e.g., thermal and high pressure processing) (Bhat et al., 2021b).

4. Lipid-based nanosystems

Different lipid-based delivery nanosystems have been developed in the past decades to be applied in the oral route to overcome the low bioavailability of bioactive compounds with poor water solubility, chemical instability and low intestinal permeability. Their main benefits, which include high biocompatibility, efficient permeation and enhanced bioavailability, are related to their capacity of stimulating biliary and pancreatic secretions, to increase the residence time in the GI tract and stimulation of lymphatic transport (Berthelsen et al., 2019).

The most common lipid-based nanosystems are nanoemulsions (NE), solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) and liposomes. NE are composed by two immiscible liquids (i.e., oil and water) and commonly stabilized with emulsifiers or surfactants (e.g., Tween 20, Tween 80, lecithin and sodium caseinate). SLN are constituted by a solid lipid in the core and stabilized with emulsifiers and surfactants, while NLC are the next generation of SLN, which are composed by a mixture of a solid lipid and a liquid oil also stabilized with surfactants or emulsifiers. Despite their similarity in terms of manufacturing, differences can be identified between SLN and NLC: the SLN are mainly utilized for the encapsulation of lipophilic compounds (i.e., they are not appropriate for hydrophilic compounds' encapsulation) whereas NLC are more appropriate to encapsulate both lipophilic and hydrophilic compounds. Furthermore, NLC are generally developed for high loadings and stability due to their non-ideal crystalline structure in the core (Katopodi & Detsi, 2021; McClements, 2018a; Nasirizadeh & Malaekeh-Nikouei, 2020).

On the other hand, liposomes are vesicular structures formed with amphiphilic molecules, such as phospholipids (Simões et al., 2017). Liposomes may present, however, some stability issues which may lead to aggregation and early drug release with subsequent degradation during storage or digestion. Nevertheless, after ingestion, lipid-based delivery systems are subjected to several physicochemical conditions, such as low pH and high ionic strength in the stomach, that may alter their structural properties (i.e. surface charge and steric coating) and their stability (Wang & Luo, 2019). For instance, triglycerides' digestion starts in the stomach due to the presence of gastric lipase, with a lower extent, but it is in the small intestine where most lipids are hydrolysed into fatty acids. This process is an interfacial process, since lipase needs to adsorb to the oil–water interface before it starts converting triacylglycerols (TAG) and diacylglycerols (DAG) into monoglycerides (MAG) and FFA (McClements, 2018b). The extension of lipase's adsorption is related to the physicochemical properties of the oil–water interface, such as interfacial structure, composition and surface area (Ye et al., 2019). Lipid digestion products and endogenous surfactants present in bile secretions (i.e., phospholipids and bile salts) can then interact via electrostatic and hydrophobic interaction and form several structures, such as unilamellar and multilamellar vesicles, and mixed micelles, where the bioactive compounds can be incorporated (Berthelsen et al., 2019; Macierzanka et al., 2019). Furthermore, bile salts have a significant interference in lipolysis, since it has been reported that their physiological function is related to the emulsification of lipids and consequently, their role significantly changes the production of micelles (Macierzanka et al., 2019). These micellar structures can enhance the transport of bioactive compounds across the mucus to the surface of the intestinal membrane and their absorption (Wang & Luo, 2019). In fact, evidence points out that the droplet size and oil type play a significant role during *in vitro* lipolysis (Salvia-Trujillo et al., 2019). In this sense, nanoemulsions composed by medium chain triglycerides (MCT) present a higher and faster free fatty acid release in the small intestine when compared with emulsions composed of long chain triglycerides (LCT). This is due to a higher water dispersibility observed for MCT containing lipids and to the fact that nanoemulsions' *in vitro* digestion results in the formation of smaller micelles that are more prone to lipase hydrolysis. However, in terms of bioactive compound bioaccessibility, e.g. LCT lipids showed a higher eugenol bioaccessibility when compared with MCT lipids, which can be related to the larger lipophilic micelle cores as a result of LCT *in vitro* digestion (Majeed et al., 2016). Moreover, Gonçalves et al. (2021) recently assessed different lipid nanosystems' digestibility (e.g., SLN, NE and NLC). The authors concluded that all nanosystems presented a fast FFA release within the first minutes of digestion with subsequent stabilization. The authors also observed that SLN presented a significantly higher FFA release at the end of the digestion process, when compared with NE and NLC, of ca. 23.8 % and 38.6 %, respectively. These differences were attributed to the nanoparticle instability and agglomeration of NE during the gastric phase (i.e., the increase in particle size promotes a lower surface area for lipase interaction and therefore, lower lipolysis) and the combination of solid lipids and liquid (i.e., in the case of NLC) which results in a higher enzymatic resistance. These nanosystems can also protect the associated tissues of the GI tract by reducing the mucosa irritation caused by continuous contact with some bioactive compounds (Madalena et al., 2019; C. Zhang et al., 2013). For instance, triptolide (TP) is known for its anti-inflammatory, anticancer, among other functional properties. However, it is also known to cause several adverse conditions to the human GI tract, namely, GI ulcer, bleeding, vomiting, mucosa irritation, among others. Regarding the irritation of the GI mucosa, it occurs due to the cellular damage caused by oxidative stress. However, the encapsulation of TP in SLN shown to have significant results in terms of reducing

mucosa irritation and this nanosystem can then be used to prevent this condition (C. Zhang et al., 2013).

Recent studies also showed that nanoemulsions' digestion is also surfactant dependent. In fact, Verkempinck et al. (2018) observed that nanoemulsions prepared with Tween 80 present a higher lipolysis rate when compared with nanoemulsions prepared with sucrose esters as surfactant. Gasa-Falcon et al. (2019) also investigated the influence of several emulsifiers (i.e., Tween 20, lecithin, sodium caseinate and sucrose palmitate), at different concentrations on the *in vitro* digestion of β-carotene nanoemulsions. The authors concluded that using low mass emulsifiers, such as Tween 20 and lecithin, produced smaller droplets when compared with sodium caseinate and sucrose palmitate, since low mass emulsifiers are capable of adsorbing to the droplets, preventing their aggregation. In fact, Tween 20 and lecithin containing nanoemulsions have shown to be more stable under gastric conditions and lecithin containing nanoemulsions partially resisted to intestinal *in vitro* digestion (i.e., around 73 % of lipid digestion). This resulted in an enhancement of the β-carotene bioaccessibility.

Proteins can also be used as surfactants and Jiang et al. (Jiang et al., 2019) recently studied the performance of pea protein nanoemulsions and nanocomplexes as delivery systems for vitamin D3. The authors showed that pH-shifting and sonication exposed some functional lipophilic amino acids which resulted in a high encapsulation efficiency of ca. 93.2 ± 2.1 %. Moreover, pea protein isolate nanoemulsions showed potential towards protecting vitamin D3 during *in vitro* gastric digestion, presenting a release of 62.9 ± 11.1 % in the small intestine phase of the *in vitro* digestion model.

Nanoemulsions can also interact with several disruptive and destabilizing constituents (Sarkar et al., 2019). From enzymes to pH transitions and shear forces (i.e., due to peristalsis), nanoemulsions can be destabilized by:

i) highly glycosylated salivary mucin via electrostatic interactions with proteins (i.e., if proteins are used as nanoemulsion emulsifiers);
ii) acidic gastric conditions that may induce a change in the nanoemulsions' structure (e.g., may cause aggregation);
iii) the proteolytic activity of pepsin, when proteins are used as emulsifiers (which interacts with protein molecules on the nanoemulsion surface, changing its interfacial properties) or interactions with gastric lipase that can cause aggregation, flocculation and coalescence, depending on the nature of the emulsifier used;
iv) the interaction with other enzymes present in the small intestine (where most of lipid digestion takes place) such as trypsin and chymotrypsin (i.e., if protein emulsifiers are used), bile salts and pancreatic lipase (i.e., responsible for the release of free fatty acids - FFA), and pancreatic amylase (i.e., if carbohydrates are used as emulsion stabilizers) (Macierzanka et al., 2019; Sarkar et al., 2019).

An example of a lipid digestion process can be seen in Fig. 1b. It is essential to study the behaviour of lipid-based delivery systems during *in vitro* digestion processes, once the stability of these systems and the nature of the micellar structures formed is determinant to understand possible mechanisms used in the solubilization and absorption of the bioactive compounds. This knowledge can then be applied to better tailor the production conditions so that these lipophilic nanosystems can meet designated application requirements.

5. Polysaccharide-based nanosystems

There has been an increasing interest in the use of polysaccharides for the formulation of bio-based nanosystems since they:

i) are natural, abundant and can be obtained from renewable sources (Dave & Gor, 2018);
ii) have high versatility since they are able to form different nanosystems depending on the applied chemical or physical processes (Dave & Gor, 2018);
iii) present important inherent biological properties such as antimicrobial, anti-inflammatory and mucoadhesive (Dragan & Dinu, 2019);
iv) have a hydrophilic character with high biocompatibility (Anda-Flores et al., 2019).

The production of polysaccharide-based nanosystems relies on chemical and biological modifications, allowing them to withstand the normal enzymatic activity and acid conditions of the upper GI tract and to act in response to a specific stimulus (e.g., changes in pH). During digestion, polysaccharides are broken down into disaccharides or monosaccharides which facilitates their absorption in the small intestine and their easier fermentation by the gut microbiota (Lovegrove et al., 2017). An example of a polysaccharide digestion process can be seen in Fig. 1c.

Chitosan is one of the most studied polysaccharides as it presents a great potential for application in the food industry. This biopolymer has antimicrobial and antifungal activities, with particular physicochemical properties responsible for its biocompatibility with human tissues and enhanced permeability (Mohebbi et al., 2019). It can be used as a controlled delivery nanosystem since it presents a slow bioactive compound release profile and high mucoadhesiveness, thus improving the bioaccessibility of bioactive compounds (e.g., polyphenols) (J. Liang et al., 2017). Recently, Guo et al. (2020) evaluated the effect of *in vitro* digestion conditions on chitosan's morphological and cytotoxicity properties. The authors observed that chitosan nanoparticles, when applied to a fasting food model (i.e., pH 7 phosphate buffer), did not dissolve during the *in vitro* digestion process, which resulted in agglomeration in the small intestine phase. In addition, chitosan nanoparticles did not significantly change the transepithelial electrical resistance (TEER) as well as cell viability, at the studied doses, when compared with the control (i.e., fasting food model). Starch and alginate are also extensively explored polysaccharides, obtained from cereals and marine algae, respectively (Ahmad et al., 2019). Studies in the literature show that these polysaccharide nanosystems present a protective effect towards bioactive compounds stability. In fact, Ahmad et al. (2019) studied the encapsulation of catechin using horse chestnut starch nanoparticles and evaluated their performance under *in vitro* digestion. The authors observed that catechin was protected against the *in vitro* gastric conditions. Free catechin presented a significant drop on its pancreatic lipase inhibition of ca. 81%, dipeptidyl peptidase IV (DPP-IV) of ca. 87.7 % and α-glucosidase of ca. 9.1 %. On the other hand, under encapsulation, catechin was able to significantly retain most of its inhibitory capabilities.

It is important to consider that most polysaccharides (except for starch and starch-based structures) are resistant to the enzymatic digestion in the upper GI tract. For instance, polysaccharides from marine algae (e.g., alginate, carrageenan, among others) are mainly digested in the colon by the microflora present there, i.e., through breaking down glycosidic bonds and consequently releasing reducing sugars that are used as substrate and consumed by the same microflora. This indicates that, besides their application in enhancing bioactive compounds bioaccessibility, such polysaccharides can also be used as prebiotic to stimulate the microflora to release beneficial compounds e.g., shot-chain fatty acids (Y. Guo et al., 2021; L. X. Zheng et al., 2020) as well as modulate its composition through increasing the bacterial growth of beneficial bacteria such as *Phascolarctos*, *Bifidobacterium, Enterococcus*, among others (Y. Guo et al., 2021). Polysaccharides can also be conjugated with other particles in order to synergistically enhance the overall nanosystem functionality (L. Dai et al., 2018). For instance, a chitosan layer can be added to β-lactoglobulin (Simões,

Martins, et al., 2020; Wei et al., 2019) and to improve the mucoadhesive properties of the nanosystem, prolonging its residence time and, consequently, the bioactive compound bioaccessibility (W. Dai et al., 2020). Polysaccharide conjugation can also be achieved with lipidic nanosystems, thus promoting emulsion stability and bioactive compound protection (Silva et al., 2019).

Notwithstanding the increasing interest and developments on polysaccharide nanosystems, their evaluation and characterization lacks in specificity, and they are often used as standalone systems without being added into a food matrix. This means that there is still a need for improvements in the development of analytical methods, which would maximize and simplify the evaluation of polysaccharide hydrolysis.

6. Current challenges of *in vitro* digestion assessment techniques

Nanosystems subjected to *in vitro* digestion can be characterized in relation to their size (Simões, Martins, et al., 2020), surface charge (He & Ye, 2019), shape (Ahmad et al., 2019), porosity (Wei et al., 2019) and rheological properties (L. Liu & Kong, 2019). Moreover, those systems can be evaluated in relation to their FFA release, if applicable, (Gonçalves, Martins, Abrunhosa, Baixinho, et al., 2021) and bioaccessibility (Bhat et al., 2021a). For this purpose, several characterization techniques have been used to assess nanosystems' behaviour under *in vitro* digestion conditions and detailed information regarding some of these techniques can be found elsewhere (Allen et al., 2019; Erdman et al., 2019; Nellist, 2019; Rahdar et al., 2019; Wiercigroch et al., 2017). This way, this review will focus on the challenges associated to using these techniques to assess nanosystems under *in vitro* digestion.

7. Particle characterization challenges

Several techniques can be employed to assess particle aggregation (Araújo et al., 2020), flocculation (B. Zheng et al., 2019) and coalescence (R. F. S. Gonçalves, Martins, Abrunhosa, Baixinho, et al., 2021), depending on the nanosystem's nature (i.e., lipidic, protein or polysaccharide). These phenomena are usually attributed to the presence of electrolytic salts, pH variation, enzymatic digestion and interactions with bile salts (Brodkorb et al., 2019; Pabois et al., 2020). To assess such behaviour during *in vitro* digestion, light scattering techniques are often applied. For this purpose, the dynamic light scattering (DLS) is the most widely used technique and it is used for routine quality control of nanoparticle production and to assess their *in vitro* digestion behaviour since:

i) it presents rapid analysis and acquisition times at reduced costs (Modena et al., 2019);
ii) it enables the measurement of a larger number of particles (when compared with microscopy measurements) (Rahdar et al., 2019), which will give a better idea regarding particle size distribution in solution;
iii) it enables particle characterization in different solvent environments (Modena et al., 2019) which is important since the *in vitro* digestion process has a complex and dynamic environment, with different conditions along the GI tract (i.e., different pH, enzymes, electrolyte concentrations and presence of bile salts).

Despite being a simple and practical technique, DLS presents some requirements and limitations that should be taken into consideration when applied to assess the performance of nanosystems under *in vitro* digestion conditions. For instance, samples must be transparent to obtain feasible particle analysis results (Modena et al., 2019; Rahdar et al., 2019; Simões, Martins, et al., 2020). However, *in vitro* digestion samples, in particular from the small intestine, present high turbidity due to the presence of, e.g., bile salts, typically from porcine bile extract, which present an orange/yellow colour as well as some potential aggregates which makes really challenging the application of DLS to analyse samples in the small intestine since its operation is highly influenced by large aggregates (Stetefeld et al., 2016). Thus, dilutions must be made in order to obtain clear transparent samples (Majeed et al., 2016; Wei et al., 2019), which may alter the overall behaviour of nanosystems. It is also important to consider that this technique is highly dependent on temperature since it will interfere with the solvent viscosity and, consequently, with the light scattering behaviour. As a result, the temperature of analysis must be controlled and constant, and the viscosity of the solvent must be known. Moreover, DLS does not differentiate particles that are close to each other, neither their shape, which makes this technique a low-resolution technique (Modena et al., 2019; Rahdar et al., 2019). Complementarily, nanoparticle tracking analysis can also be used to study and assess size (i.e., from ca. 50 nm to 1000 nm), distribution and concentration. This technique combines DLS with dynamic microscopy to track individual particles in solution by analysing the centre of the light scattered when it interacts with the particles and recording each nanoparticle trajectory (Gross-Rother et al., 2020). This way, a real-time visualization of the nanoparticles' movements is possible and, adjusting the analysis parameters (e.g., refractive index) may lead to the identification of different nanoparticle aggregates which can make this approach more suitable for polydisperse samples (Gross-Rother et al., 2020; van der Pol et al., 2010). On the other hand, since this technique can be dependent on the analysis settings, it can consequently be more subjective since it may depend on the user interpretation. Furthermore, the analysis of protein particles can pose a challenge due to their low refractive index and consequently low scattered light (Gross-Rother et al., 2020).

Nanosystems can also be classified regarding their morphology since their shape is linked to their functionality, environmental response and bioactive compounds' release kinetics (Mourdikoudis et al., 2018). This way, the morphological characterization of nanosystems is one of the most important assessment procedures to evaluate their properties and behaviour. Some of the most used conventional morphology characterization techniques are transmission electronic microscopy (TEM), scanning electronic microscopy (SEM), atomic force microscopy (AFM), fluorescence microscopy and confocal laser scanning microscopy (CLSM) where SEM and TEM are the most used techniques to assess the size and shape of nanosystems and, as such, are often used in combination with scattering techniques (e.g., DLS) (Modena et al., 2019), and fluorescent and CLSM microscopy can be used to assess nanoemulsions and detect droplet coalescence throughout the digestion process (R. F. S. Gonçalves, Martins, Abrunhosa, Vicente, et al., 2021). Studying the morphological changes that occur during the *in vitro* digestion process provide a more realistic idea regarding particle aggregation, size and shape, when compared with standalone analytical techniques such as scattering techniques (e.g., DLS) (Falsafi et al., 2020). However, in the context of *in vitro* digestion, some challenges can be identified regarding this assessment. For instance, sample manipulation is often needed to perform such analysis (e.g., sample dilutions, surface coatings, among others) (Vladár & Hodoroaba, 2020) which can interfere with the performance of nanosystems under *in vitro* digestion conditions. Furthermore, it is important to take into consideration that microscopic techniques are destructive assessment techniques that only represent a portion of the analysed sample.

7.1. Rheological characterization

Rheology is a well-established science of the deformation and flow of matter. Rheological properties are obtained by relating the stress applied on a material and the subsequent deformation as a function of time. Nowadays, with the advances in instrumentation, food rheology not only plays a crucial role in measuring apparent viscosity, but also provides in-depth information on microstructure and fluidity of a food matrix, allowing the assessment of the network structure integrity (Mandala & Apostolidis, 2020).

Nanosystems' rheological properties play a crucial role in modifying

the texture of foods and in their performance during *in vitro* digestion. Few studies have been conducted regarding the assessment of apparent viscosity of nanosystems during the different digestion phases aiming at a better understand of their behaviour in the GI tract and their effects on food digestion and nutrient absorption (Liu & Kong, 2019). This information can be used to tailor the production of functional food products. For instance, studying the changes in viscosity during gastric digestion can lead to the conclusion that the application of high viscosity materials can promote stomach emptying retardation and consequently, an increasing perception of satiety (Espert et al., 2019). In fact, Espert et al., (2019) observed that higher amounts of xanthan gum and consequently, higher apparent viscosity, promoted a significant decrease on the amount of oleic acid released from cream digestion. Rheological studies can also be used to indirectly assess the integrity of intermolecular bonds between nanoparticles during the *in vitro* digestion process and their resistance to the hydrolytic effect (Pabois et al., 2020). Recently, Liu & Kong, (2019) studied the influence of different types of nanocellulose (NC) on whey protein isolate digestion. For this purpose, the authors used cellulose nanofibrils (CNF), TEMPO-oxidized CNF and cellulose nanocrystals (CNC) and observed that the digesta viscosity was positively correlated to the concentration of CNF and CNC which resulted in lower whey protein isolate hydrolysis through lowering the initial free amino nitrogen diffusion rates by ca. 31.4 % and 68.4 % in the case of CNF and CNC respectively.

There are some limitations that can be identified regarding the application of rheological studies as a tool for assessing nanosystems during *in vitro* digestion. For instance, the complex environment of an *in vitro* digestion poses a major challenge towards data interpretation since several interfacial phenomena occurs during digestion (Zornjak et al., 2020) and isolating such phenomena is quite challenging. Furthermore, the rheological assessment can be a time consuming and destructive process (i.e., since it is a contact technique) that is very difficult to apply as an *in situ* analytical tool since it will interfere with the digestion process and micro-rheology can be used for this purpose. Micro-rheology is a rheological technique that uses particle tracers as probes and advanced imaging and processing techniques to study complex structures (e.g., gel-like structures), particles' interfaces, among others. As such, the mean square displacement of the tracer is measured over time which will give important information regarding the viscoelastic properties of the sample (Xia et al., 2018). Different particle probes (e.g., fluorescent, magnetic) can be used for this purpose and more detailed information regarding this subject can be found in the work of Xia et al., 2018. The application of micro-rheology to the digestion process can give important insights regarding, e.g., the interfacial properties of nanoemulsion during the digestion process (Yang et al., 2021), the trajectory of nanoparticles using fluorescent tracers (Xia et al., 2018), among others. When compared with traditional rheology, micro-rheology presents a higher sensitivity which makes it a potential technique to identify the effects and phenomena that occur during the *in vitro* digestion of nanosystems. Despite its potential, the application of micro-rheology to assess nanosystems during the digestion process is still very limited and very few studies are found in the literature regarding this subject.

8. Non-conventional *in vitro* digestion assessment techniques

The assessment of nanosystems under *in vitro* digestion can also be achieved by non-conventional techniques to further unravel the structural changes that occur in such systems and understand the phenomena associated to the digestion process. Methods such as small-angle X-ray scattering (SAXS), Raman spectroscopy, fluorescence resonance energy transfer (FRET) and nuclear magnetic resonance spectroscopy (NMR) can be applied for this purpose.

SAXS is a technique that can be used to perform structural analysis (e.g., particle size, shape, dispersity, morphology) of polymeric molecules in solution. This way, an X-ray beam is transmitted and passes through the sample. The elastically scattered electrons are then collected, at a small angle, by a two-dimensional detector to form a SAXS image which is then processed. Subsequently, each pixel of the image is converted into the scattering angle. Thus, different scattering angle patterns can determine the structural characteristics of a given sample (Brotherton et al., 2019; Lv et al., 2018). For instance, Lv et al., (2018) applied this technique, *in situ,* to understand the structural changes of lipidic microemulsions during *in vitro* digestion as well as the influence of the mucus on lipolysis. The authors observed the formation of liquid crystalline phases during lipolysis which were subsequently damaged in the presence of the mucus, which is attributed to the hydrophobic interactions between the crystalline structures and the mucus. This technique can be further improved by coupling it with a synchrotron, improving the scattering intensity of the radiation (Franke & Svergun, 2020; Khan et al., 2016). This method can be used to assess the development of colloidal structures during lipolysis in real-time. In fact, this assessment was conducted by Khan et al., (2016), which evaluated the precipitation and the solid-state form of lipidic nanoemulsions (i.e., composed by fenofibrate). The authors concluded that fenofibrate precipitates in its thermodynamically stable crystalline form during lipolysis. Therefore, SAXS techniques can be used to study the structural changes that occur during the *in vitro* digestion of nanosystems, allowing a better understanding regarding their fate and digestion kinetics (e.g., bioactive compounds' release rates, enzymatic activity, among others) in the GI tract. Furthermore, smaller scattering angles can be used, i.e., ultra-small angle x-ray scattering (USAXS), to perform a wider colloidal analysis since it enables the detection of particles from 300 nm to ca. 2000 nm, when compared to SAXS (up to 100 nm) (Sakurai, 2017). Therefore, this technique has the potential to be used in *in vitro* digestion studies to assess, for instance, nanoparticle interactions during the digestion process (e.g., particle aggregation, coalescence, among others) since it can analyse a wider range of particle sizes. However, SAXS techniques are not widely available (i.e., especially synchrotron SAXS), since they require expensive equipment, maintenance and highly specialized knowledge regarding signal processing and data interpretation (Franke & Svergun, 2020; J. Li et al., 2018).

Raman spectroscopy is a vibrational spectroscopic technique that uses monochromatic light to extract information regarding the molecular vibrational modes (i.e., it correlates with the inelastic scattering of photons) and transitions which will in turn allow inferring about intermolecular interactions (Deidda et al., 2019; Salim et al., 2020). This methodology is a fast, non-destructive and eco-friendly technique that requires little to no sample preparation prior to analysis and it can be used for qualitative determinations such as to assess food quality (W. Zhang et al., 2020), deterioration (R. Hu et al., 2019) and fraud detection (Berghian-Grosan & Magdas, 2020), as well as for quantitative studies such as fermentation monitoring (A. Zhang et al., 2020) or compound quantification (Chen et al., 2019). Under *in vitro* digestion conditions, Raman spectroscopy can be applied to analyse, in real-time, bioactive compounds' solubilization (Salim et al., 2020) and crystallization (Stillhart et al., 2013). However, this approach needs to be coupled with powerful analytical techniques for spectra interpretation and thus, chemometric approaches (e.g., Partial Least Squares Regression, Principal Component Regression) (Zhiming Guo et al., 2019; W. Zhang et al., 2020) and machine learning (artificial neural networks, support vector machines, among others) (Berghian-Grosan & Magdas, 2020; Z. Zhang, 2020) have been used for this purpose.

Despite the advancements in the application of Raman spectroscopy for the assessment of nanosystems' performance under *in vitro* digestion conditions, these studies are still very scarce and information on dynamic *in vitro* digestion models is still very limited, perhaps due to their dynamic nature which will interfere and lower the signal to noise ratio of Raman spectra (Deidda et al., 2019). Furthermore, care must be taken when applying this technique for *in vitro* digestion assessment since inconsistent results were frequently observed regarding e.g., compounds' solubilization when compared to other techniques (e.g., SAXS)

(Salim et al., 2020).

FRET is a physical phenomenon that occurs when the energy of an excited fluorophore donor is transferred to an acceptor fluorophore through dipole–dipole interactions. Thus, this technique requires that the two fluorophores must be near to each other (i.e., at a distance between ca. 1 to 10 nm) (Zhiming Guo et al., 2019). This principle can be used to study the interfacial properties of emulsions (Pan & Nitin, 2016) and their interactions with other constituents (e.g., mucus) under *in vitro* digestion conditions (Lv et al., 2018). Pan & Nitin (2016) assessed, in real-time, the dynamics of a lecithin emulsion (with and without chitosan coating) interface under *in vitro* intestinal digestion conditions. The authors observed that the phospholipids in the emulsion interface were immediately disrupted by the addition of bile salts. This disruption was significantly prevented by the addition of a chitosan layer which implies a lower rate of FFA release. Lv et al. (2018) also used FRET to investigate the fate of a self-microemulsifying drug delivery system under *in vitro* digestion conditions. As previously mentioned, the authors used SAXS and observed that the initial liquid crystalline phases disappeared. However, FRET measurements enabled the authors to conclude that the interaction between the emulsion and the mucus formed micelles from the liquid crystalline phases. Therefore, FRET can be used as a standalone or combined technique to investigate the structural changes that may occur to bioactive compounds' delivery systems under *in vitro* digestion conditions.

NMR spectroscopy is also a potential technique to assess nanosystems during the digestion process. Briefly, this technique uses a magnetic field that takes advantage of the magnetic properties of molecular nuclei (typically ^{1}H, ^{13}C and ^{31}P – proton NMR) which are then subjected to radiation in the radiofrequency (RF) region of the electromagnetic spectrum (Hatzakis, 2019). This technique can then give important qualitative (i.e., the position of the spectrum peaks determines the molecular structure of the samples) and quantitative (i.e., the area under the peaks is proportional to the number of nuclei responsible for that peak) information regarding the chemical composition of samples and, consequently, it can be applied to monitor nanosystems' digestion by analysing the release of metabolites during the digestion process (Smeets et al., 2021). It can be used to assess the lipolysis of nanoemulsions (Nieva-Echevarría et al., 2016, 2017), protein hydrolysis (Deng et al., 2022) and conformational changes (Jain & Sekhar, 2022), among others. However, some challenges can be identified which include low sensitivity and high cost, and it requires in-depth NMR knowledge regarding theoretical and spectra interpretation. Moreover, a standard protocol addressing sample preparation is still required for this technique to be generalized along with the publication of NMR spectra databases so that comparisons and statistical analysis can be made in complex samples (Hatzakis, 2019).

9. *In vitro* digestion protocol challenges

Food digestion is a dynamic and very complex process, with several pH transitions, different ionic strengths and several intervenient compounds (i.e., enzymes, salts and bile salts), which interfere with the behaviour of nanosystems under each of these conditions. These interferences should not be ignored, and care must be taken while performing the intended analysis. Therefore, several challenges can be highlighted when evaluating the behaviour of nanosystems throughout the *in vitro* digestion process (C. Li et al., 2020).

During *in vitro* digestion, several samples are usually taken to determine the overall digestion kinetics and the integrity of the nanosystem or the bioactive compounds' release kinetics at the end of each digestion stage. Consequently, the enzymatic digestion must be stopped, or at least attenuated, in order to obtain reliable results at a specific sample time. Commonly, samples are kept at low temperatures (i.e., often through ice baths or liquid nitrogen) to stop further enzymatic reactions. However, the low temperature conditions can influence the overall nanosystem dynamics and its structure, as well as the release kinetics of bioactive compounds (C. Li et al., 2020; Pinheiro et al., 2017). For instance, some proteins present some degree of denaturation at low temperatures which can cause protein unfolding and aggregation, and consequently the release of bioactive compounds (Arsiccio et al., 2020). Other techniques to prevent the enzymatic digestion of the samples may include the addition of sodium hydroxide (NaOH) or sodium bicarbonate ($NaHCO_3$) to raise the pH and stop gastric enzymatic digestion and the addition of Pefabloc® SC to block the activity of trypsin and chymotrypsin (Mulet-Cabero et al., 2020). However, raising the pH of digestion samples may induce unwanted phenomena such as particle aggregation due to the isoelectric point of the nanoparticles which, in turn, can interfere with the digestion results.

Dynamic *in vitro* digestion systems can be used to estimate the bioaccessibility of bioactive compounds, in particular the models that encompass a filtration process in jejunum and ileum stages such as the TIM-1 model (Dupont et al., 2018). Their dynamic classification is often attributed based on the control of their pH (e.g., gradual pH adjustment in the gastric phase), stomach emptying behaviour, fluid injection over time (i.e., electrolytes, enzymes and bile salts), as well as the presence of peristalsis (Lucas-González et al., 2018). In order to achieve this last feature, plastic bags are often used as containers for the digestion process, which are alternately contracted to simulate GI peristalsis. However, some bioactive compounds present some affinity to plastics and adsorb to their surface (e.g., carotene) (Berni et al., 2019) and this must be taken into account when calculating the release kinetics of bioactive compounds under GI conditions. For instance, Berni et al (2019) reported a change in colour of the plastic bags (i.e., colour changed to a light yellow colour and light red colour for buriti and pitanga emulsions, respectively) due to the affinity of carotene to plastic, especially when in an aqueous medium. This results in an underestimation of bioaccessibility of carotene in the intestinal phase.

Despite the existing challenges regarding the assessment of bio-based nanosystems under *in vitro* digestion conditions, several *in vitro* digestion models have been used for this purpose and the latest developments and challenges are reviewed in the next topic.

10. *In vitro* digestion models

10.1. Current trends and challenges in the development of novel in vitro digestion models

When compared to *in vivo* options, *in vitro* digestion models are low cost systems without any ethical constraints that provide the opportunity to understand the behaviour of nanosystems and bioactive compounds release kinetics under digestion conditions in a more or less controlled environment, depending on the complexity of the model (i.e., static, semi-dynamic or dynamic) (Brodkorb et al., 2019; Pinheiro et al., 2017). Regarding this subject, several detailed reviews can be found in the literature describing different *in vitro* digestion models (Ji et al., 2021; C. Li et al., 2020; Mackie et al., 2020), thus this review will focus on the latest developments in the field.

The COST INFOGEST group introduced a standardized static *in vitro* digestion protocol which is based on *in vivo* physiological data that allows inter-laboratory comparisons between assays (Minekus et al., 2014). This initiative was a significant landmark in the field since, until then, comparing *in vitro* digestion results was limited to studies that used the same *in vitro* digestion conditions/models (e.g., within the same research group) and no inter-laboratory comparisons could be made. More recently, this standardized protocol was updated by Brodkorb et al. (2019) by introducing the presence of gastric lipase in the gastric phase of the protocol, which was not commercially available previously. In fact, there is a current awareness from the scientific community to develop standard physiologically relevant *in vitro* models, that can be applied by a wide range of research groups since the use of standard laboratory equipment is a major concern while developing such protocols (Brodkorb et al., 2019).

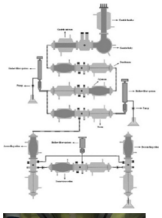
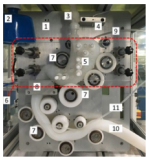
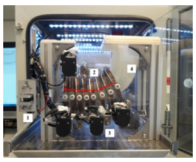
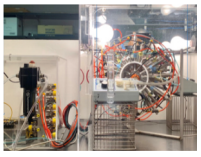
Despite of their simplicity, static *in vitro* digestion models have been widely used to study the digestion of nanosystems in the upper GI tract (Gonçalves, Martins, Abrunhosa, Vicente, et al., 2021; Simões, Martins, et al., 2020; Xu et al., 2021), since they can be used to study the influence of the chemical conditions of the GI tract. However, more complex and realistic kinetic studies should be performed under dynamic *in vitro* digestion conditions (Brodkorb et al., 2019), since the former models lack the dynamic nature of the human digestive system regarding pH gradients, gastric emptying and physiologically relevant GI motility behaviour. On this regard, Mulet-Cabero et al. (2020) recently developed a standardized *in vitro* semi-dynamic model that takes into consideration the gradual pH changes that occur during food digestion.

Despite of the current awareness related to the standardization of *in vitro* digestion models, much work must be done since the current standardized models still do not consider nutrient absorption and intestinal dynamic behaviour. Moreover, some important mechanical grinding phenomena (e.g., retropulsion) and pyloric filtering are not considered and thus, dynamic models are required for such studies. The most recent advances by the scientific community were devoted to the development of novel *in vitro* dynamic digestion models (mostly *in vitro* gastric models), more specifically, to mimic the anatomical, mechanical and physiological behaviour of the human stomach (Y. Li et al., 2019; W. Liu, Fu, et al., 2019; Vrbanac et al., 2020; J. Wang et al., 2019) and small intestine (J. Wang et al., 2019), in addition to simulating the physicochemical conditions of the GI tract – Table 1.

Dynamic *in vitro* digestion models were recently applied to study the digestion of protein (Bourbon et al., 2018), lipid (Machado et al., 2019; Silva et al., 2018) and polysaccharide (Silva et al., 2019) digestion. Furthermore, the most recent dynamic *in vitro* digestion models simulate the anatomical shape and size of the human stomach (i.e., a J-shaped structure), through the application of 3D printing technologies to develop the mould of the stomach and small intestine and by the application of deformable elastic materials. Materials like silicone (Liu et al., 2019) and latex (Li et al., 2019) have been used for this purpose since they are elastic, deformable, non-toxic, chemically resistant and inert materials (Vrbanac et al., 2020; Wang et al., 2019). Moreover, some novel digestion models simulate the inner surface roughness, conferred by the inner surface folds in the human stomach lumen (Li et al., 2019; Wang et al., 2019), aiming at representing a more realistic dissolution behaviour and grinding forces. Due to the "J" shape of the human stomach and the simulation of the pyloric sphincter, phenomena like retropulsion may take an important role in food grinding and consequent particle sieving, since the pylorus only allows the passage to the duodenum of particles with a diameter below 1–2 mm (Li et al., 2019; Vrbanac et al., 2020).

Simulating the human GI peristalsis has been a concern since it takes a crucial role in food grinding. The simulation of the GI peristaltic movements has been achieved through different approaches. For example, Wang et al. (2019) used a roller system to contract the *in vitro* stomach and small intestine; Li et al. (2019) applied multiple inflated pneumatic syringe systems at different locations to simulate the human stomach contractions; Liu et al. (2019) used a belt system to apply contraction forces to the *in vitro* stomach model; and Vrbanac et al. (2020) simulated the peristaltic contractions through a "worm gear"

Table 1
Recent advancements regarding the development of novel *in vitro* digestion systems. (Images reprinted, Copyright 2022, with permission from Elsevier and the Royal Society of Chemistry).

Model	Digestion phases	Peristalsis simulation	Performance assessment	Schematic representation	Reference
Gastrointestinal simulator system	Gastric, small intestine and large intestine compartments	Water jacked reactors	Mixing time; Gastrointestinal pressure; Gastric crushing force.		(Z. Li et al., 2019)
Dynamic *in vitro* human stomach	Oesophagus, gastric and duodenum compartments	Roller system	Gastric emptying of beef stew; *In vitro* digestion of cooked rice; Gastric retention ratio.		(J. Wang et al., 2019)
Artificial gastric digestive system	Stomach	Roller belt system	Gastric force; *In vitro* digestion of α-lactalbumin;		(W. Liu, Fu, et al., 2019)
Advanced gastric simulator	Stomach	Worm gear system	Table dissolution; Gastric emptying flows.		(Vrbanac et al., 2020)
Gastric simulation model	Stomach	Pneumatic squeezing syringes	Measurement of intragastric pressure; *In vitro* gastric digestion of cooked sausage; Gastric diffusion and emptying of methylene blue affected; Gastric emptying of amberlite beads.		(Y. Li et al., 2019)
Soft robotic gastric simulator	Gastric Compartments	Inflated soft robotic system	Contraction forces; Contraction ratio;	NA	(Dang et al., 2020)

constriction mechanism. Due to the versatility of *in vitro* digestion models, some can even simulate the GI conditions of population specific groups (e.g., infants and elderly). For instance, infant *in vitro* digestion models are often composed only by the gastric and intestinal phases, i.e., the oral phase of digestion is not included mainly due to the lower α-amylase levels and low swallowing times (i.e., the main source of nutrients comes from milk). These models present higher gastric pH of ca. 5 which lowers pepsin digestion (i.e., optimal pH ranging from 1.6 – 4) but promotes gastric lipase activity (i.e., optimal pH ranging from 3 – 5) which retains its activity throughout the GI tract (i.e., from pH 1.5–7), playing an important role during the intestinal lipolysis (Mackie et al., 2020). Similar to the infants, the elderly population also presents higher pH values in the stomach (ca. 4), however, they present higher concentrations of α-amylase (ca., 150 U/mL) during the oral phase of the digestion (Mackie et al., 2020; Shani-Levi et al., 2017) and lower bile salt concentration (ca. 5.14 mmol.L^{-1}) in the intestinal phase (Hernández-Olivas et al., 2020; Mackie et al., 2020).

Different approaches have thus been used to simulate the real conditions in the human stomach and small intestine, in terms of anatomy, surface roughness, mechanical behaviour, population specific conditions and sieving capabilities. However, work needs to be done to accommodate the current differences between the existing *in vitro* digestion models and *in vivo* systems. For instance, the presence of mucus in the stomach and gastric selective absorption behaviour could take an important role towards simulating human digestion and this way, obtaining more realistic predictions of food digestion kinetics (C. Li et al., 2020). Moreover, efforts should be done towards standardizing dynamic *in vitro* digestion models so that interlaboratory studies and comparisons could be made. This way, one should expect that the next step towards the standardization of *in vitro* dynamic models would be the development of easy to replicate dynamic models through the application of, e.g., 3D printing to develop both the digestion compartments and peristatic mechanisms. To the authors knowledge, there is still a lack of standardized *in vitro* digestion models that simulate the GI conditions of population specific groups (e.g., elderly and infants). However, efforts have been made towards this path with the development of a potential standard *in vitro* infant digestion protocol by Ménard et al., (2018). Moreover, the validation of *in vitro* digestion models is still a major challenge (Ketnawa et al., 2021; Li et al., 2020) and while several papers reviewed the recent developments on digestion models (Gonçalves et al., 2021; Ji et al., 2021; Li et al., 2020), very few discuss the *in vitro* digestion models' validation which is the focus of the next topic.

11. Validation of *in vitro* digestion models

It is clear from the previously discussed topics that, regarding the development of new dynamic *in vitro* digestion models, different approaches and materials have been used to simulate the digestion conditions of the human GI tract. However, none of the models developed so far are truly validated (Ketnawa et al., 2021; C. Li et al., 2020). In fact, from the analysis of Table 1, it can be inferred that several strategies were used to assess the performance of those models which implies that no standard protocol has not yet been developed to validate such models. It is thus important to discuss what should be considered for their validation, the challenges associated to the validation process as well as some possible solutions.

The validation is perhaps the most complex step in the development of novel *in vitro* digestion models. Therefore, several questions arise while addressing this topic such as: What is a validated model? Which are the current challenges that limit their validation? Which are the possible solutions than can contribute to their validation? Is it possible to have a fully validated model?

One should expect that a fully validated model would be able to predict the bioaccessibility of nutrients and bioactive compounds with lower errors and deviations from *in vivo* data, i.e., with higher accuracy, when compared to unvalidated models. However, the models' validation will be dependent on their final application, e.g.:

i) the assessment of a specific food product's digestion;
ii) the simulation of the GI tract of a specific population (e.g., regional, continental, age specific);
iii) the simulation of the GI tract of specific species (i.e., simulation of the GI tract of mice);
iv) the assessment of specific GI pathological conditions;
v) the bioaccessibility assessment of food products and bioactive compounds.

Therefore, a model should only be validated if the *in vivo* validation data used can significantly represent its final application, i.e., if an *in vitro* digestion model is designed to represent the GI tract of a specific population (e.g., population of a given country or region), then the *in vivo* data from the same population should be used for its validation (Shani-Levi et al., 2017). Of course that obtaining representative *in vivo* digestion data is still a major challenge due to ethical constrains, complexity and cost (Pinheiro et al., 2017). In fact, the complexity of the human digestive system poses a major challenge due to high inter and intra-individual variability, e.g., the concentration of enzymes, transit time, gastric emptying rate, among others that vary with the individual's diet, sex or age (Eker et al., 2020; C. Li et al., 2020). Consequently, comparing the statistical difference between *in vitro* and *in vivo* data would not be appropriate. Furthermore, the scarcity of *in vivo* studies, especially regarding the assessment of nanosystems, and the reduced number of test subjects per study (e.g., human or animal test subjects) could pose a challenge towards the representativity of the *in vivo* data in the studied population.

Despite the identified challenges, some strategies could be applied to solve or at least attenuate their consequences. For instance, the development of an open access world-wide database composed, initially, by *in vivo* digestion data of predetermined, standard food products/nanosystems could be a solution towards increasing sample size and consequently, sample representation (Shani-Levi et al., 2017). For such development, it should be investigated which parameters are more appropriate to be considered and measured *in vivo*. Such research would result in the development of a standardized *in vivo* digestion protocol which would be crucial for the comparison of data obtained world-wide, i.e., interlaboratory comparison. With the development of a world-wide database, sophisticated algorithms, e.g., artificial neural networks, random forests, genetic algorithms, support vector machines, cluster analysis, among others, could be used to identify patterns in the *in vivo* data. The same approach could also be used to analyse the data variability among individuals which would result on identifying the patterns related to the *in vivo* data variance. This error pattern identification could be further used to correct the *in vitro* digestion data which would increase the *in vitro-in vivo* correlations.

Still, there is a lot of work to be done and world-wide efforts must lean towards the validation of *in vitro* digestion models, either through the development of standard protocols regarding the collection and assessment of *in vivo* data or through the application of technologically advanced analytical techniques to unravel data trends and better correlate *in vitro* and *in vivo* digestion data.

12. Concluding remarks and future perspectives

The assessment of nanosystems as controlled delivery systems of bioactive compounds under *in vitro* digestion conditions is of utmost importance to understand their fate in the GI tract. The knowledge of their behaviour under simulated *in vitro* conditions allows the optimization of nanosystems' design and production taking into consideration their performance under *in vitro* digestion, so that material dosage and processing can be adjusted. Protein, polysaccharide and lipid-based nanosystems have been used as bio-based protective vehicles of

bioactive compounds, showing promising results regarding their protective performance under harsh digestion conditions. However, efforts must be made to further enhance nanosystems' performance in terms of expanding their functional properties and protective ability, as well as understanding their digestion mechanisms and bioactive compounds' release kinetics. It is also important to take into consideration that, despite all the advantages of delivery systems at the nano scale, some concerns remain unanswered regarding the safety and, consequently, the application of nanosystems to food products and their consumption. Therefore, regulatory agents must address this issue to overcome this bottleneck. More information regarding this topic can be found on the detailed works of M. Wang et al. (2021). To assess the performance of nanosystems under digestion conditions, it is crucial to develop reliable analytical methods for particle size, dispersibility and surface charge determination, as well as for morphological, rheological, electrophoretic, free fatty acid, bioaccessibility (and consequently, bioavailability) and cytotoxicity assessment. Despite their routine application, care must be taken while performing such analysis since the digestion process involves the presence of several factors that can interfere with the assessment of nanosystems' behaviour (e.g., presence or absence of peristalsis, stomach emptying, gastric pyloric sieving, among others). Therefore, advances in the development of reliable and realistic *in vitro* digestion models are crucial so that better correlations with *in vivo* data can be obtained.

There is a current effort by the scientific community to standardize *in vitro* digestion protocols, so that interlaboratory comparisons can be established, as well as to develop anatomical accurate dynamic *in vitro* digestion models with realistic peristaltic contractions, inner surface rugosity, pyloric sieving and stomach emptying. This way, it is expected that more robust, generalized and complex *in vitro* digestion systems will be developed to obtain accurate results regarding nanosystems' digestion behaviour. However, the validation of *in vitro* digestion models is still a challenge due to the complexity of *in vivo* studies.

The fast-paced increase of research regarding the *in vitro* digestion field can lead to the development of more sophisticated real-time analytical tools, so that many of the challenges discussed in this review can be overcome. Furthermore, with the development of novel, accurate and sophisticated analytical tools and *in vitro* digestion models, more opportunities in the field of *in silico* analysis could arise so that resources could be spared. The development of an open access word-wide database containing data related to *in vivo* studies could catalyse the creation of a standardized *in vivo* digestion protocol and consequently the creation of an *in vitro* digestion model validation assessment through the application of advanced analytical techniques (e.g., artificial intelligence).

Therefore, a lot of work must be done towards improving *in vitro-in vivo* correlations by taking into consideration e.g., the presence of a stomach mucus layer and gastric absorption, as well as by including cellular models within *in vitro* digestion systems, so that bioactive compounds' absorption can be assessed *in situ* and, therefore, avoiding sample manipulation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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