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In vitro digestion models to evaluate lipid based drug delivery systems; present status and current trends

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ABSTRACT

During the past two decades, a range of *in vitro* models simulating the digestion processes occurring in the stomach and small intestine have been developed to characterize lipid based drug delivery systems (LbDDSs). This review describes the presently existing range of *in vitro* digestion models and their use in the field of oral drug delivery. The models are evaluated in terms of their suitability to assess LbDDSs, and their ability to produce *in vitro - in vivo* correlations (IVIVCs).

While the pH-stat lipolysis model is by far the most commonly utilized *in vitro* digestion model in relation to characterizing LbDDSs, a series of recent studies have shown a lack of IVIVCs limiting its future use. Presently, no single *in vitro* digestion model exists which is able to predict the *in vivo* performance of various LbDDSs. However, recent research has shown the potential of combined digestion-permeation models as well as species specific digestion models.

Contents

Abbreviations: AUC, area under the curve; BS, bile salt; BCS, biopharmaceutics classification system; BSA, bovine serum albumin; DDS, drug delivery system; DG, diglyceride; DGM, dynamic gastric model; FA, fatty acid; GI, gastro-intestinal; HLB, hydrophilic-lipophilic balance; HTP, high-throughput lipolysis model; IVIVC, *in vitro - in vivo* correlation; LbDDS, lipid based drug delivery system; LFCS, lipid formulation classification system; MG, monoglyceride; PL, phospholipid; PK, pharmacokinetic; rHGL, recombinant human gastric lipase; SEDDS, self-emulsifying drug delivery system; SMEDDS, self-micro-emulsifying drug delivery system; TIM-1, TNO gastro-intestinal model 1; TG, triglyceride; UWL, unstirred water layer.

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1. Introduction

In the field of oral drug delivery, *in vitro* models are commonly used in the pharmaceutical development phase to estimate *in vivo* drug performance and optimize formulation design prior to preclinical and clinical studies. For this purpose, numerous *in vitro* models have been designed to simulate key processes related to drug absorption, *e.g.* dissolution, digestion, and permeation across an absorption barrier. The models vary greatly in their level of complexity and physiological relevance; ranging from simple one step, one-compartment models to multi-compartmental models simulating several processes simultaneously [1,2]. In the case of the simpler *in vitro* models, simulating a single process, the general idea is to mimic the rate-limiting step to drug absorption [3]. If several processes occur simultaneously and affect each other, *e.g.* drug release from a supersaturating system and drug permeation, more complex *in vitro* models are needed to simulate all processes simultaneously. When *in vitro* models are used to predict *in vivo* behavior, they should be designed to mimic the *in vivo* situation as closely as possible. However, as *in vitro* models are generally desired to require a low labor intensity, be rapid, robust, and cheap, the final design is typically a compromise between complex and simple. Due to the complexity of the human body, there is no perfect *in vitro* model for evaluating *in vivo* drug performance, and the design and selection of appropriate *in vitro* models should always be made based on prior knowledge of the human physiology, the drug delivery system (DDS) in question, and the physicochemical characteristics of the specific drug [4].

In vitro models simulating gastro-intestinal (GI) digestion are widely used to study the behavior of food and pharmaceuticals during GI transit [5–8]. During the past decades, several thousand research articles using *in vitro* digestion models have been published; the majority in the field of nutrition, and approximately 700 dealing with drugs [6]. To characterize digestible DDSs, a range of *in vitro* models have been developed to simulate the digestion processes occurring in the human stomach and small intestine [9–28]. The present review describes this range of *in vitro* digestion models and their use in the field of oral drug delivery. The models are evaluated in terms of their suitability to assess lipid based drug delivery systems (LbDDSs), and their ability to produce *in vitro - in vivo* correlations (IVIVCs). Trends for future *in vitro* digestion models are also presented, and evaluated similarly. Design, development, and classification of LbDDSs, as well as the human digestion processes have previously been extensively reviewed [6,8,29–35], and are therefore, only summarized shortly below.

2. Lipid based drug delivery systems

Due to contemporary drug discovery programs, the majority of new drug candidates, intended for oral administration, display poor water solubility, and therefore, require enabling DDSs to reach the desired bioavailability and thereby therapeutic effect [36]. One group of enabling DDSs designed to bypass a slow dissolution process and increase the apparent drug solubility in the GI fluids, is LbDDSs. LbDDSs comprise a relatively wide range of physically different systems including lipid solutions, self-emulsifying DDSs (SEDDSs), and micellar systems [31,32]. Most LbDDSs present the drug to the GI tract in solution, *i.e.* solubilized in lipids, surfactants, and co-solvents, or mixtures thereof [29,31,32]. Based on the choice of excipients, LbDDSs can be categorized into four classes according to the lipid formulation classification system (LFCS) originally proposed by Pouton et al. (Table 1) [32,33]. Although some shortcomings of the LFCS have been pointed out, *e.g.* that it does not apply to all marketed LbDDSs, and that it does not provide an indication of the likely *in vivo* performance [31,37], the LFCS, by and large, serves as a simple and useful classification system to distinguish between the abundance of LbDDSs. According to the LFCS, Type I formulations are characterized by consisting solely of lipids, being non-dispersing in aqueous media, and requiring digestion to release encapsulated drug. Type II formulations are isotropic mixtures of lipids and lipophilic surfactants (with a hydrophilic-lipophilic balance (HLB) <12) forming SEDDSs when introduced in aqueous media. Type III formulations are also SEDDSs or self-micro-emulsifying DDSs (SMEDDSs), but contain hydrophilic rather than lipophilic surfactants (HLB > 12) and/or hydrophilic co-solvents. Type IV formulations are very hydrophilic, made purely from surfactants and hydrophilic co-solvents. Upon dispersion, the water-soluble surfactants and the hydrophilic co-solvents can dissolve into the surrounding aqueous media, which means, that type IV formulations have a high risk of losing solvent capacity upon dispersion, resulting in a risk of drug precipitation [32,38].

Table 1
The lipid formulation classification system (LFCS).

Excipients in formulation	Content of formulation (%, w/w)				
	Type I	Type II	Type IIIa	Type IIIb	Type IV
Oils: triglycerides or mixed mono and di-glycerides	100	40–80	40–80	<20	–
Water-insoluble surfactants (HLB < 12)	–	20–60	–	–	0–20
Water-soluble surfactants (HLB >12)	–	–	20–40	20–50	30–80
Hydrophilic co-solvents (*e.g.* PEG, propylene glycol, transcutol)	–	–	0–40	20–50	0–50

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Despite their diversity, LbDDSs share a range of biopharmaceutical, pharmaceutical and commercial advantages [29]. Of the greatest importance are the biopharmaceutical advantages; *i.e.* drugs administrated in LbDDSs are mostly presented to the GI tract in solution, and thereby bypass the possible rate limiting drug dissolution step, facilitating drug absorption [29,31,39]. Upon digestion, the solvation capacity of a LbDDS is typically reduced which induces supersaturation and potentially results in drug precipitation [40–42]. However, if the drug does not precipitate, *e.g.* when the activation energy associated with crystal formation is high or in the presence of a polymeric precipitation inhibitor, the increased saturation will increase the free concentration of drug in equilibrium with the solubilized reservoir, and thereby increase drug absorption [42]. Aside from bypassing the drug dissolution step, as well as potentially increasing drug absorption driven by drug supersaturation, the presence of lipid excipients, surfactants and co-solvents has also been shown to increase drug permeation through the intestinal mucosa by inhibiting the P-gp efflux pump [43–48], opening tight junctions [49–51], and increasing membrane fluidity [43,52]. In addition, a high content of long chain lipids will delay gastric emptying, and also stimulate lymphatic uptake of lipophilic drugs (for drugs with logP >5, and lipid solubility >50 mg/g) [53,54].

A few key challenges associated with LbDDSs, are directly related to their main biopharmaceutical advantages. As previously mentioned, following dispersion and/or digestion, the solvation capacity of the LbDDS (or the digested LbDDS) is commonly reduced which may lead to drug precipitation [29,33,55]. With drug precipitation, the advantage of administrating the drug in solution is (to some degree) lost, and with it the potentially increased drug absorption. If the drug precipitates in the amorphous form, it might re-dissolve quickly, and thereby limit the negative implications of drug precipitation as illustrated in Fig. 1 [56,57]. As the risk of drug precipitation increases with drug load [37,42,55], the development of a new LbDDS includes a trade-off between avoiding drug precipitation and maximizing drug load and promoting supersaturation to drive drug absorption. In addition to the risk of drug precipitation, the use of surfactants to increase drug solubilization may decrease the free fraction of drug, resulting in decreased drug absorption. As illustrated in several studies by *e.g.* Dahan et al., it is important to consider the solubility-permeability interplay of micellar DDSs, as increased drug solubilization *in vitro* and/or *in vivo* does not necessarily lead to increased drug absorption [58–66].

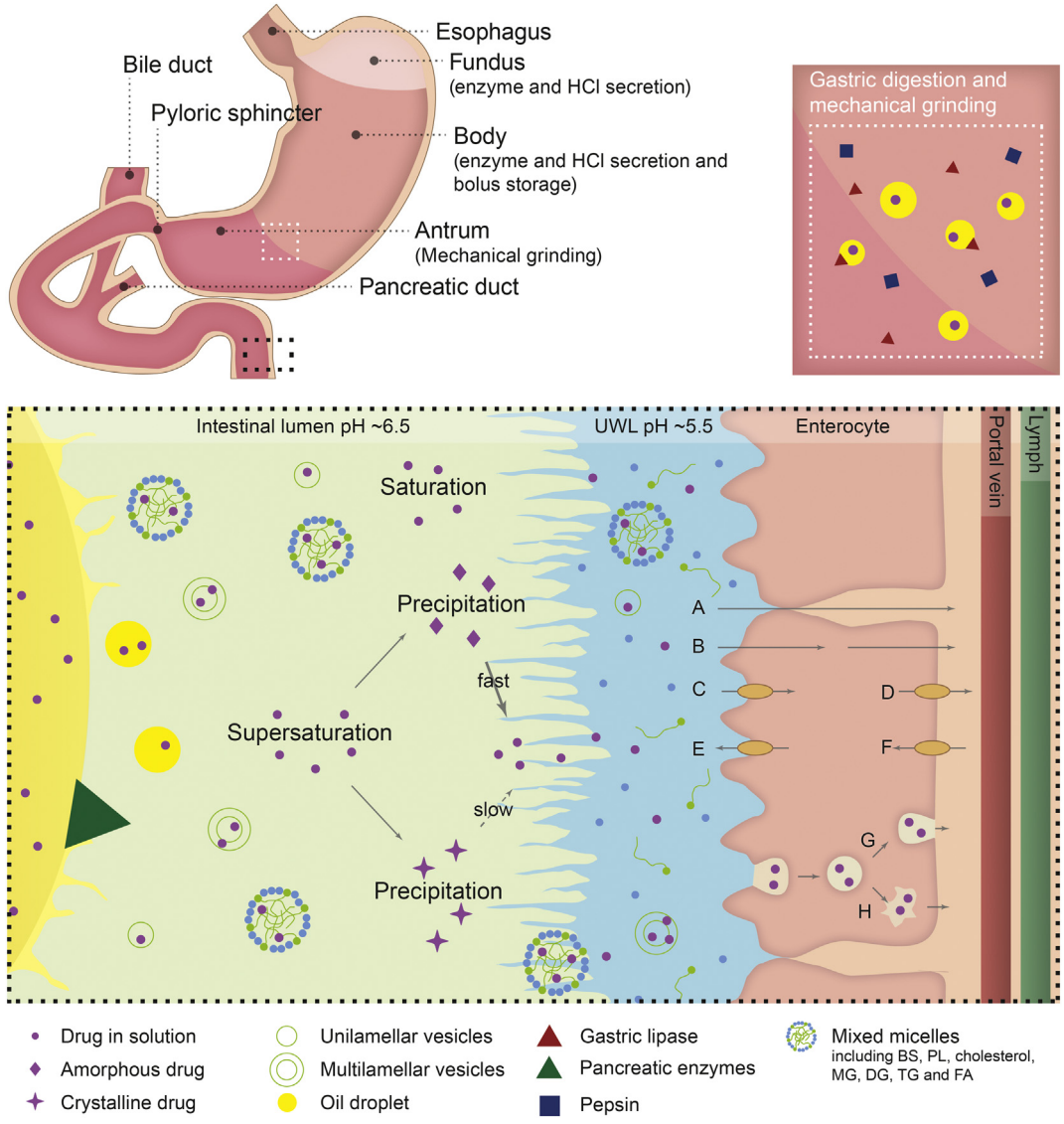


Fig. 1. Schematic presentation of lipid digestion, drug solubilization, and absorption processes occurring in the stomach and small intestine. The stomach, the duodenum and the initial part of the small intestine are illustrated in the top left corner with a zoom into the lower part of the stomach and the intestinal lumen, to the right and below, respectively. Transport pathways across the epithelial layer: paracellular passive diffusion (A), transcellular passive diffusion (B), influx/efflux facilitated transport by membrane proteins (C—F), transcytosis (G) and endocytosis (H). Abbreviations: bile salt (BS), phospholipids (PL), monoglycerides (MG), diglycerides (DG), triglycerides (TG), fatty acids (FA) and unstirred water layer (UWL). Components are not drawn to scale.

3. Human gastro-intestinal digestion

Human GI digestion includes two main processes occurring simultaneously, *i.e.* mechanical and enzymatic digestion [6]. The digestion processes begin in the mouth and continue through the GI tract to the large intestine. In the mouth, ingested food is masticated, mechanically broken down and mixed with saliva to form a soft, swallowable mass termed the bolus. Saliva consists of approx. 99% water, < 1% minerals, and 0.1–0.2% proteins, including the digestive enzyme amylase which initiates the enzymatic digestion process [8,67]. After the bolus is swallowed, it passes through the esophagus to the stomach, which can be separated into three regions; the fundus, the body and the antrum (Fig. 1). Acidic gastric juices, containing pepsin and gastric lipase, of which the latter is responsible for digesting 5–40% of ingested triglycerides (TGs) [68–72], are secreted from the fundus and the body of the stomach, which also serves as a reservoir. In the antrum, the bolus is subjected to further mechanical stress, before the semi-fluid mass of partly digested food, now called the chyme, leaves the stomach to enter the duodenum. In the duodenum, the chyme is mixed with bile and pancreatic juice for additional digestion prior to entering the jejunum and ileum [73]. Bile secretions contain endogenous surfactants in the form of bile salts (BSs) and phospholipids (PLs), which remove lipolysis products from the lipase-substrate interface and stabilize emulsified lipid-droplets, thereby aiding lipid digestion [70]. The pancreatic juice contains lipases, amylases, and proteases, which support the enzymatic digestion of the different components of an ingested meal. In addition, the pancreatic juice holds a high concentration of bi-carbonate ions, which neutralizes the acidic fluid arriving from the stomach [74,75]. During the course of lipid digestion, various colloidal phases are formed *e.g.* unilamellar vesicles, multilamellar vesicles and mixed micelles (Fig. 1) [18]. The mixed micelles composed of BSs, PLs, and lipolysis products in the form of mono-glycerides (MGs), and free fatty acids (FAs), are expected to diffuse through the unstirred water layer (UWL) to the surface of the intestinal membrane where the different constituents are absorbed [76]. The mechanism behind the dissociation of the lipolysis products from the mixed micelles is presently not fully understood, however, it has been suggested, that the process is associated with an acidic microclimate found in the unstirred water layer

covering the intestinal membrane [77–79]. According to the microclimate theory, ionized FAs are converted to their non-ionized forms upon entering the acidic microclimate, and as a result of their reduced solubility in the mixed micelles, the FAs dissociate from the micelles and are rapidly absorbed across the intestinal membrane [77]. Any material which is not digested and absorbed within the small intestine, passes to the large intestine where different anaerobic bacteria have the capacity to *e.g.* ferment carbohydrates and proteins, and metabolize xenobiotics [80].

Fig. 1 illustrates some of the lipid digestion, drug solubilization, and absorption processes occurring in the stomach and small intestine. The free fraction of dissolved drug can permeate the UWL covering the intestinal wall, and be absorbed across the epithelium. When the free drug concentration is above the limit of saturation, *i.e.* supersaturated, the drug might precipitate in either a crystalline or an amorphous form. Precipitated drug needs to dissolve again before it can be absorbed. The colloidal structures improve passage of the drug across the UWL and present the drug to the apical membrane at the brush border of the enterocytes. Different transport pathways are available across the epithelium: paracellular passive diffusion, transcellular passive diffusion, influx/efflux facilitated transport by membrane proteins, transcytosis and endocytosis. Once the drug has passed the basolateral membrane, it will be transported through the portal vein or, in case of a lipophilic drug (logP >5, lipid solubility >50 mg/g), *via* the intestinal lymphatic system, to the systemic circulation [29,53,76,81].

Table 2
Overview of the most commonly considered physiological effects taken into account in the various digestion models. Abbreviations: dynamic gastric model (DGM), TNO gastro-intestinal model (TIM-1), and high-throughput lipolysis model (HTP).

	DGM	TIM-1	HTP	pH-stat lipolysis model		
				Intestinal	Gastro-intestinal	
					Immediate transfer	Continuous transfer by pump
	Gastric conditions					
pH[a]	+	+	−	−	+	+
Enzymatic conditions[b]	+	+	−	−	+	+
Transit time	+	+	−	−	+	+
Gastric emptying	+	+	−	−	−	+
Mechanical digestion	+	+	−	−	−	−
	Intestinal conditions					
pH[a]	−	+	+	+	+	+
Enzymatic conditions	−	+	+	+	+	+
Mechanical digestion	−	+	−	−	−	−
Absorption	−	−	−	−	−	−

[a] Constant or pre-programmed.
[b] Non-human gastric lipase.

4. *In vitro* digestion models

Ideally, when simulating the complex physiological and physicochemical events occurring in the human GI tract following oral administration of a LbDDS, each step of the digestion processes should be mimicked with realistic pH and enzymatic conditions, transit times, and mixing [34,82]. Additionally, resident microbiota, the immune system, feedback mechanisms and specific hormonal controls should be accounted for, in order to accurately simulate human digestion [34]. Furthermore, simulation of the uptake mechanism in the small intestine, lymphatic transport, as well as hepatic metabolism might also have to be mimicked to ensure accurate prediction of the *in vivo* behavior of LbDDSs. Unfortunately, simulating all of these complex multistage processes is technically very difficult, time consuming, and costly. Therefore, based on the assumption, that a simplified digestion model can capture the relevant effects of human GI digestion in relation to the oral performance of LbDDSs, a series of simplified *in vitro* digestion models have been developed. These models vary in their level of complexity, number of compartments, as well as in which elements of the human digestion they simulate. Table 2 shows an overview of the physiological effects taken into account in the most commonly used *in vitro* digestion models, *i.e.* the pH-stat lipolysis model, the dynamic gastric model (DGM), the TNO gastro-intestinal model (TIM-1), and the high-throughput lipolysis model (HTP). In the following sections, the presently existing range of *in vitro* digestion models, which have been used to evaluate LbDDSs, are presented and discussed in terms of suitability to predict *in vivo* performance. The models are grouped according to whether they simulate only enzymatic digestion, or simulate both enzymatic and mechanical digestion. The models are presented with an increasing level of complexity. In the last section, trends for future *in vitro* digestion models are described, including patient specific models and combined digestion-permeation models.

4.1. Models simulating enzymatic digestion

The most widespread *in vitro* digestion models used to evaluate LbDDSs are simulating only enzymatic digestion. The models typically mimic the intestinal (or the gastric and the intestinal) phase of digestion, using physiologically relevant media designed to mimic the GI fluids including digestive enzymes at relevant activities. All studies are performed at 37 °C, commonly at a fixed pH. Within the food sciences, digestion in the oral cavity is commonly included in the *in vitro* models [6,34,83]. However, with respect to LbDDSs, which are swallowed directly following administration, the residence time in the oral cavity is considered irrelevant, and this phase is therefore omitted from *in vitro* digestion models used to characterize LbDDSs.

The majority of the enzymatic digestion models used to evaluate LbDDSs are variations of the pH-stat lipolysis model [9–25]. In the following sections, the concept of the pH-stat lipolysis model is described, alongside its various modifications. Additionally, a high-throughput intestinal lipolysis model, the HTP model, designed to enable a fast evaluation of LbDDSs without the use of pH-stat titration [84,85], is described.

4.1.1. pH-stat lipolysis models

The most frequently used *in vitro* digestion model for evaluation of LbDDSs is the pH-stat lipolysis model depicted in Fig. 2. The model represents a relatively simple and cheap setup, which can be used for LbDDS screening purposes. The experimental setup most commonly comprises one single compartment used to simulate intestinal digestion [12,13,15–20,86–91]. The *in vitro* digestion takes place in a thermostated reaction vessel with the tested LbDDS dispersed in a digestion medium resembling fasted or fed state intestinal fluid, prepared with an appropriate pH, buffer capacity, as well as concentrations of BSs and PLs simulating the effects of bile secretion in the duodenum. Digestion is initiated by manual addition of digestive enzymes, simulating the addition of pancreatic juice. Pancreatic lipase and other esterases present in porcine pancreatic extract, which is the most commonly used source of pancreatic lipase and found to be very similar to human pancreatic extract [7,92,93], hydrolyze TGs and other digestible LbDDS excipients, which subsequently release free FAs [94]. The degree of digestion is indirectly determined from the amount of sodium hydroxide required to neutralize the pH drop caused by the enzymatic hydrolysis. Calcium is added, either continuously throughout the digestion experiment (the dynamic lipolysis model) or as an initial bolus (the static lipolysis model), to precipitate the FAs released during digestion [92]. If the FAs are not removed from the digestion medium, they will build up at the emulsion interface and inhibit further digestion [95]. Overhead stirring is used to mix the digestion media, enabling homogenous sampling. During *in vitro* digestion, three distinct phases are typically formed (Fig. 2); an oil phase consisting of undigested, non-

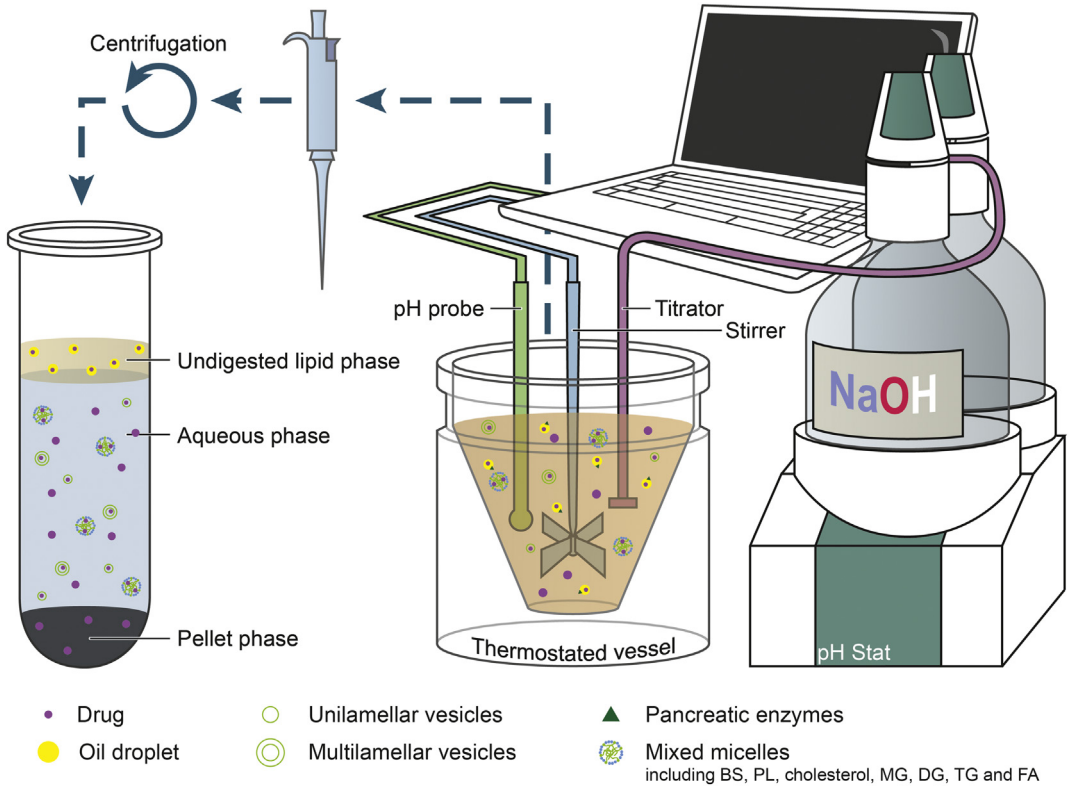


Fig. 2. pH-stat lipolysis model for *in vitro* assessment of lipid-based drug delivery systems. Abbreviations: sodium hydroxide (NaOH), bile salt (BS), phospholipid (PL), monoglyceride (MG), diglyceride (DG), triglyceride (TG), fatty acid (FA).

emulsified lipids, an aqueous phase containing small vesicles and micelles, and a pellet phase comprised of precipitated drug, calcium soaps of FAs, and digestive enzymes [7]. Immediate following sampling, the enzymatic activity is inhibited, *e.g.* by addition of a lipase inhibitor, in order to stop the digestion processes at the given sample time point [7,15]. Based on the hypothesis, that the amount of drug solubilized in the aqueous phase is available for absorption, quantification of the drug content in each of the digestion phases, will allow the prediction of pre-absorption oral performance of LbDDSs. Furthermore, by quantifying the amount of drug in each separate phase, as well as in a so-called "total sample" representing all the phases thoroughly mixed together, is it possible the perform mass balance calculations and thereby evaluate the accuracy of the measurements, which is typically reported to be relatively high (> 90%) [26,40].

The exact composition of the digestion medium, including the choice and level of BSs and PLs, the choice of lipase source and level of activity, the manner of calcium addition, as well as the digestion medium volume, varies between different research groups [7,8,38,83,94]. Each of these *in vitro* model setup variations has been shown to impact on the *in vitro* digestion results, and has been thoroughly discussed in various review papers [7,8,38,83,94], for which reason, the effects will not be repeated here. In general, the experimental variables are set to mimic the *in vivo* situation as close as practically possible. However, due to the complexity and dynamic behavior of the human GI tract, it is impossible to closely mimic all processes occurring simultaneously. Therefore, all the designed versions of the pH-stat lipolysis models may be considered physiologically relevant, and no one model stands out over the others. During the past decade, the LFCS Consortium, representing an industry-academia collaboration, has conducted a large number of studies towards the establishment of standardized *in vitro* tests for LbDDSs [37,41,55,92,96,97]. As variations in the *in vitro* model setup can have significant effects on the *in vitro* performance of LbDDSs, model variations often prohibit inter-laboratory comparisons, for which reason a standardized *in vitro* model is considered beneficial. The work conducted by the LFCS consortium entails the description of baseline conditions to conduct *in vitro* digestion tests, as well as studies on the effect of *e.g.* different types and concentration of BSs, calcium amount and addition, drug load, as well as the level of pancreatic extract utilized during digestion experiments [37,41,55,92,96,97].

4.1.1.1. Gastro-intestinal digestion. For most purposes, a one-compartment intestinal digestion model is considered adequate; however, to evaluate the impact of low pH and gastric digestion, as well as gastric emptying and sudden pH-changes, a simple intestinal digestion model will not be sufficient [98]. For that reason a two-step one-compartment, as well as a two-step two-compartment pH-stat lipolysis model have been developed, allowing simulation of both gastric and intestinal digestion, using the pH-stat lipolysis setup [24–26]. In the two-step one-compartment GI digestion model, the gastric digestion phase is simulated by dispersing the LbDDS in simulated gastric fluid, and adding gastric enzymes. Following a fixed period of gastric digestion, a concentrated intestinal medium and a pancreatic enzymatic solution are added to obtain a final digestion medium composition representative of the intestinal fluids. During both the gastric and the intestinal phase, the pH is kept constant by automatic titration of sodium hydroxide, and between the two steps, the pH is adjusted from gastric pH to intestinal pH [24,25]. In the two-step two-compartment GI digestion model, two reaction vessels and two pH-stat apparatuses are used in series simulating the stomach, and small intestine, respectively (Fig. 3). Similar to the intestinal digestion model and the two-step, one-compartment GI digestion model, simulated gastric and intestinal fluids are used in the two-compartmental GI digestion model. Following initial dispersion of the LbDDS in the gastric medium, and addition of gastric digestive enzymes, the digestion medium is continuously pumped from the gastric compartment to the intestinal compartment containing a concentrated intestinal medium and pancreatic enzymes. The rate of the GI transfer is set to mimic the gastric emptying pattern, thereby

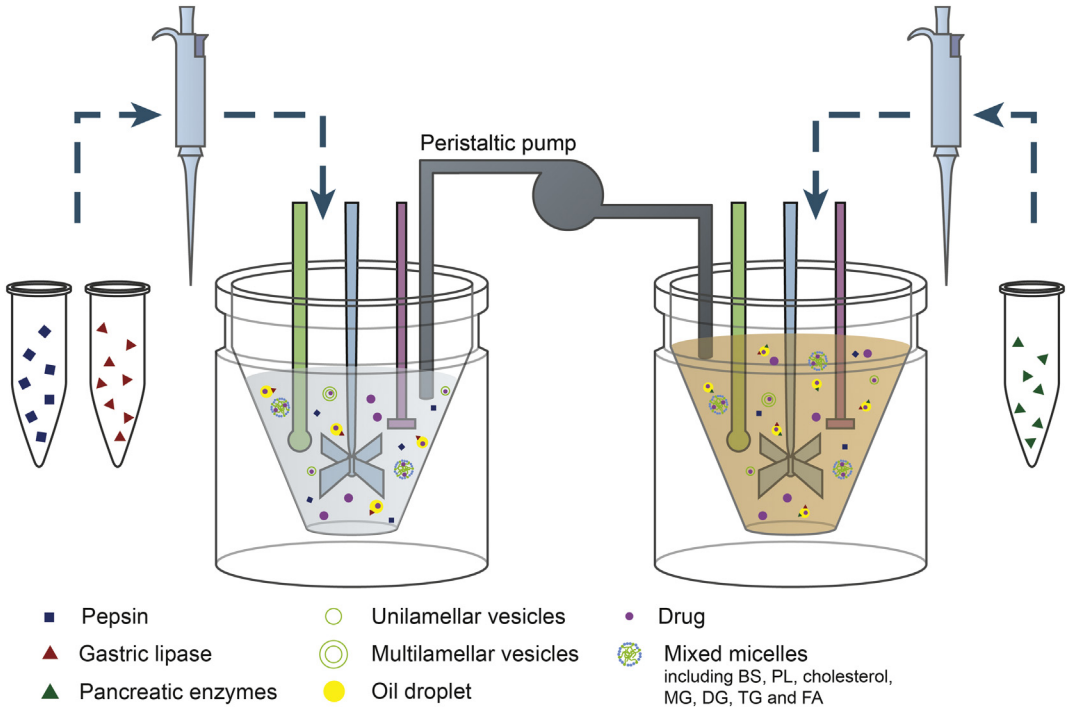


Fig. 3. Schematic overview of the two-step two-compartment GI digestion model. The transfer rate can be set to vary over time to simulate the gastric emptying, using a peristaltic pump. Abbreviations: bile salt (BS), phospholipid (PL), monoglyceride (MG), diglyceride (DG), triglyceride (TG), and fatty acid (FA).

mimicking the *in vivo* situation more closely as compared to the one-compartment model [26,99,100].

Whilst the porcine pancreatic extract is well accepted as a substitute for human pancreatin [7,92,93], substituting human gastric lipase has proven to be more difficult. Most commonly, microbial or animal derived lipase is used to stimulate gastric digestion [24–26,101]. However, as the pH-optimum, the substrate affinity and the stereo selectivity of human gastric lipase is different from that of microbial and animal derived lipases [101], no good substitute for human gastric lipase presently exists. A newly developed recombinant human gastric lipase (rHGL) may have potential [101]; however, as it has only recently become commercially available, it has yet not been incorporated into any gastric lipolysis model evaluating LbDDSs. As a consequence of the limited availability of a human gastric lipase, *in vitro* hydrolysis of lipids in the gastric compartment is likely not to be predictive of what is observed *in vivo*. This is a problem, especially when evaluating substrates highly susceptible to human gastric lipase such as *e.g.* the non-ionic surfactant Labrasol [102].

4.1.1.2. In vitro –in vivo correlations of the pH-stat lipolysis model. Establishing IVIVC for a LbDDS is a complicated challenge. As *in vivo* lipolysis studies are yet to be conducted, a direct comparison between *in vitro* and *in vivo* data does not exist. As an alternative, *in vitro* solubilization data are commonly related to *in vivo* absorption data. These correlations are based on the general consensus that drug solubilization is the rate limiting step to absorption of drugs with a high permeability and a low water solubility, *i.e.* the biopharmaceutics classification system (BCS) class II drugs [3]. With this, and the assumption that a high aqueous solubilization *in vitro* equals a high bioavailability, the amount of drug solubilized in the aqueous phase during *in vitro* digestion studies, has been related to the output of *in vivo* pharmacokinetic (PK) studies, *i.e.* the maximum plasmaconcentration, C_{max}, the time to reach the maximum plasmaconcentration, t_{max}, and the area under the curve, AUC.

Despite a series of studies demonstrating the potential of the pH-stat lipolysis models by establishing rank-order IVIVCs [12,13,17,24,90,103], an equal number of studies has since revealed a lack of IVIVCs using the same digestion models [19,24,58,104–107]. In a recent review by Feeney et al., it was pointed out that an IVIVC was obtained for 4 out of 8 tested drugs (Danazol, Griseofulvin, CP-532,623, and Torcetrapib *vs* Fenofibrate, Dexamethason, Cinnarizine and E804), but that no physicochemical drug property was found to explain the likelihood of a correlation [29]. The lack of IVIVCs has been suggested to be caused by the assumption that a high aqueous micellar solubilization and a low degree of precipitation during *in vitro* digestion studies, is equal to a high bioavailability [38]. As the pH-stat lipolysis models only take pre-absorption processes into account, ignoring possible *in vivo* events, such as transport through the gut wall, excipient efflux pump inhibition, lymphatic transport, excipient efflux pump inhibition, hepatic first pass-metabolism and saturable enzyme degradation, the assumption that a high aqueous micellar solubilization and a low degree of precipitation during *in vitro* digestion studies equals a high bioavailability *in vivo*, might indeed be wrong. Still, it is important to stress, that an IVIVC is usually obtained in the cases of LbDDSs for which the amount of solubilized drug in the aqueous phase following *in vitro* lipolysis is high, whereas it varies whether an IVIVC is obtained or not, for LbDDSs showing drug precipitation during *in vitro* digestion tests [29]. This means, that the pH-stat lipolysis model could be used as a very conservative screening model, rejecting poor formulation candidates, however, also rejecting some promising ones.

In relation to simulating the *in vivo* situation as closely as possible, the pH-stat lipolysis model has several drawbacks. Of key importance are the following three; i) The model lacks an absorption step, which means that during *in vitro* digestion, lipolysis products build up at the emulsion interface and subsequently inhibit further digestion, and that drug in a supersaturated state might precipitate [95]. To avoid inhibition by lipolysis product build-up, calcium is added prior to initiation of the experiment, or continuously throughout the experiment, as calcium forms insoluble soaps with FAs and thereby removes them from the system [92]. The lack of drug removal from the possibly supersaturated digestion medium, will most likely lead to an overestimation of drug precipitation, which in turn may produce a wrong estimation of the *in vivo* drug performance. ii) With respect to the GI lipolysis models, the pH-stat lipolysis model setup is unsuited for quantifying gastric digestion, as FAs are typically unionized at a pH below 3, and therefore cannot be titrated [104,108]. Additionally, as previously mentioned,

the limited commercial availability of a lipase with similar digestive properties as human gastric lipase further hampers the *in vitro* studies on the effect of gastric digestion on the bioavailability of LbDDSs [101]. iii) The overhead stirring does not accurately mimic the hydrodynamics of the GI tract. For the evaluation of LbDDSs which easily emulsify, overhead stirring is generally considered sufficient to ensure homogeneous sampling, however, in the case of LFCS type 1 formulations, which do not (easily) disperse in aqueous media, simulating the *in vivo* mixing may have a crucial effect on the predicted drug release.

Despite the mentioned drawbacks of the pH-stat lipolysis model, and the lack of consistent IVIVCs, the use of the model, often in combination with solid state and colloid characterization, has greatly increased the understanding of how lipophilic drugs traffic during digestion of LbDDSs [7,94,109]. However, in terms of predicting the bioavailability or oral performance of LbDDSs, the models are often falling short.

4.1.2. The high throughput lipolysis model

With the purpose of developing a high-throughput *in vitro* lipolysis model for a faster evaluation of LbDDSs, Mosgaard et al. designed a model without pH-stat titration, *i.e.* the HTP model [84,85]. The HTP model was originally tested in thermostated reaction vessels similar to those used in the pH-stat lipolysis model, however, it was also tested in 96-well plates to increase the throughput and down-scale the material [84]. To avoid pH-stat titration, the digestion medium utilized for the HTP model was based on a different buffer system (Bis-Tris) at a higher concentration (200 mM), ensuring a high enough buffer capacity to diminish the pH drop associated with lipid digestion [84,85]. For evaluation, the HTP model was compared to the dynamic intestinal pH-stat lipolysis model. Three LbDDSs (LFCS class I-III) loaded with cinnarizine or danazol were tested in both models, comparing i) the pH profiles during digestion, ii) the amount of drug solubilized in the aqueous phase, and iii) the amount of digested TGs quantified using high pressure liquid chromatography. In both experimental setups, the HTP model was found to be equivalent to the dynamic pH-stat lipolysis model [84,85,110], showing promise for improving the throughput and cost-effectiveness of the pH-stat lipolysis model. However, as the HTP model has not been validated with regards to *in vivo* data, and as it represents a simplification of the pH-stat lipolysis model, it is expected that it shares the same shortcomings, and will not be able to consistently predict the *in vivo* performance of LbDDSs.

4.2. Models simulating preprocessing and mechanical (and enzymatic) digestion

As previously mentioned, most *in vitro* digestion models, designed to evaluate LbDDS, focus on the enzymatic digestion processes. In contrast, simple dispersion tests in which no enzymes are added, can be considered as simple *in vitro* models that only simulate the LbDDS preprocessing occurring during gastric mechanical digestion *in vivo*. Additionally, the dynamic gastric model (DGM) and the TNO gastro-intestinal model 1 (TIM-1), originally designed for food research, are suitable to study both, enzymatic digestion, and the effect of *e.g.* gastric emptying rate and the GI hydrodynamics on LbDDSs [105,111–114]. Both, the DGM and the TIM-1 model, are relatively complex and are therefore not suitable for large-scale formulation screening purposes.

4.2.1. Dispersion tests

The dynamic dispersion test conducted in a standard dissolution testing apparatus (typically USP 2), represents the simplest way of simulating the GI solubilization and mechanical digestion processes. The test is typically performed in 200–250 mL of simulated gastric or intestinal media, to represent the dilution process following oral administration with *e.g.* a glass of water [105]. The dispersion media are stirred to simulate the physiological mixing occurring in the GI tract, and samples are taken throughout the study, filtered and analyzed to determine the amount of drug in solution [105]. The results from the dispersion test are used to estimate the likelihood of drug precipitation upon mixing and dilution in GI fluids, not taking the effects of enzymatic digestion into account [29].

In some cases, this simple dispersion test has produced IVIVCs, which was unattainable using the intestinal pH-stat lipolysis model [105,106], however, in other cases the dispersion test did not lead to an IVIVC [115]. Due to the inconsistent results in terms of obtaining IVIVCs, the dispersion test is not considered suitable to estimate the *in vivo* performance of LBDDSs.

As the *in vivo* estimates obtained using the dispersion test appear to be equally good/bad as the those obtained using the pH-stat lipolysis model, the need to study the effects of enzymatic digestion while estimating the *in vivo* performance of LbDDSs can be questioned. However, as none of the models in question has been able to produce reliable *in vivo* predictions across a range drugs and LbDDSs, it is difficult rank one model over the other.

4.2.2. The dynamic gastric model

The DGM is a gastric digestion model developed to simulate both the mechanical and the biochemical aspects of gastric digestion in a realistic time-dependent manner [116,117]. The DGM was originally designed for food-based research, however, as the model represents a very realistic tool for the replication of human gastric digestion, it has since been widely used for both food, and pharmaceutical applications [111,118–122].

Fig. 4 shows a schematic overview of the DGM comprising two sections, the main body and the antrum, simulating different parts of the human stomach. The upper part, the funnel, simulates the fundus and the body, and serves as a reservoir for the bolus. The funnel is made up by a cone-shaped elastic membrane, which allows rapid heat transfer to and from the surrounding water bath, as well as gentle agitation applied by a variable external pressure. The addition of acid and gastric enzymes is computer-controlled, and occurs through a perforated hoop placed at the top of the main body. The gentle agitation ensures that the outer layer of the bolus is wetted by the simulated gastric secretions, which decreases the viscosity of this layer as compared to the rest of the bolus, and allows for migration to the antrum [116,117]. The lower part of the DGM simulates the antrum part of the stomach. It consists of a barrel and a moving piston, enforcing shear forces so that the mixing and the grinding of the bolus occurs in a similar manner to *in vivo* [123]. Gastric emptying is mimicked by sequentially emptying chyme through an outlet valve. As the DGM only simulates gastric digestion, the secreted chyme needs to be transferred to a different model, *e.g.* a pH-stat controlled intestinal digestion model, to evaluate the effects of intestinal digestion [116,117].

The key advantage of the DGM is the close replication of the mechanical stress which ingested food and pharmaceuticals are exposed to in the human stomach. In addition, the DGM allows the use of complex food matrices similar to those used in clinical studies, *e.g.* the FDA breakfast [116]. With regard to LbDDSs, the DGM has been used as a characterization tool to investigate the physical processes involved in the emulsification of SEDDSs prepared from soybean oil, Tween80, and Span80 in the ratio 65:17.5:17.5 (*w*/w/w) [111]. The mechanical stress of the DGM resulted in 5–10 fold smaller droplets (2–6 μm) compared to the droplets obtained when using a standard USP II paddle apparatus (11–33 μm). The results indicate that the mechanical agitation affects the droplet size, despite SEDDSs being self-emulsifying systems. In addition, an increase in droplet size was observed for the last fraction of gastric contents being emptied from the DGM, indicating either a poor initial emulsification, or that the formed emulsion was unstable [111]. As the droplet size will affect the surface area available for digestion and drug release, the results obtained by Mercuri et al., indicate the importance of mimicking the mechanical digestion during *in vitro* evaluation of LbDDSs. However, as results obtained using the DGM have not been compared to results obtained in a purely enzymatic digestion model and evaluated towards *in vivo* data, it is presently impossible to

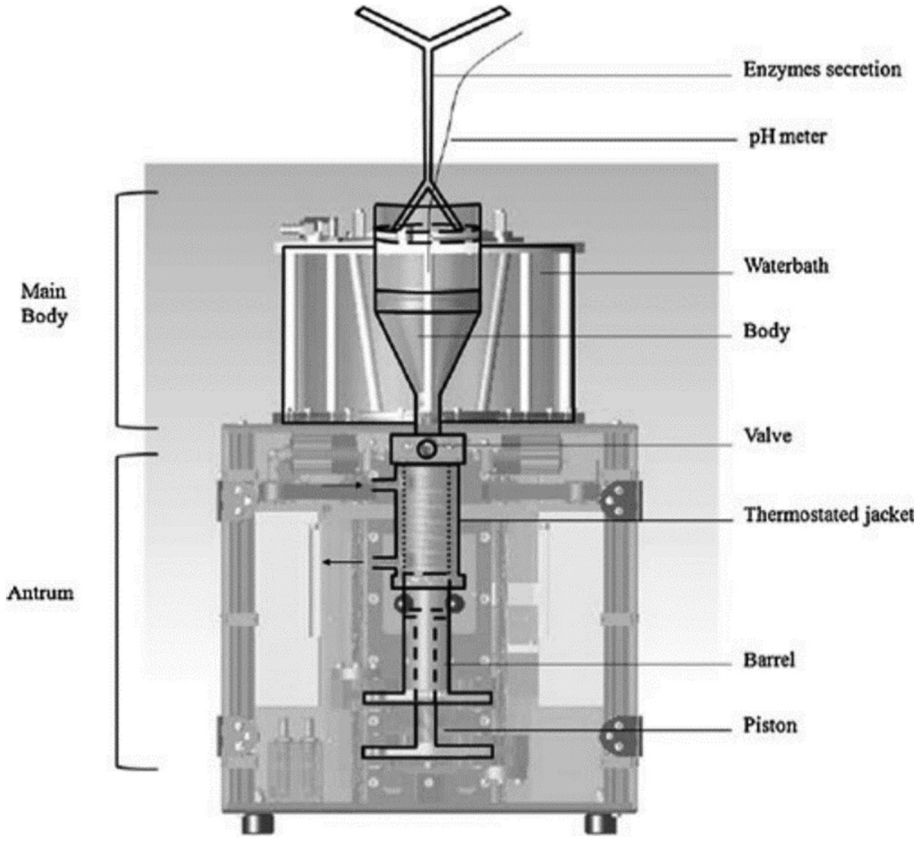


Fig. 4. Schematic overview of the DGM divided into two sections; the main body and the antrum. Reprinted from [111] with permission from Springer Nature (2019).

state if mimicking the gastric mechanical digestion processes increases the *in vivo* predictability of *in vitro* digestion models.

The biochemical aspects of gastric digestion are simulated in the DGM by addition of acid, gastric pepsin of porcine origin and microbial lipase derived from Rhizopus Oryzae (ROL) [116,117]. As previously mentioned, microbial lipase is not an optimal substitute for human gastric lipase, and consequently, the hydrolysis of lipids in the DGM is likely not predictive of what is observed *in vivo*.

The DGM has not yet been evaluated in relation to predicting the *in vivo* performance of LbDDSs. It has, however, been evaluated in bioequivalence studies of controlled release formulations, as well as tablet disintegration and capsule rupture studies, showing good IVIVCs [111,117,118,124]. Based on this, the DGM is considered a good tool to simulate the mechanical stress LbDDSs experience in the stomach. However, in order to mimic the enzymatic gastric digestion more closely, the presently used gastric lipase (ROL), should be replaced by *e.g.* rHGL.

4.2.3. The TNO gastro-intestinal model

The TIM-1 is a multi-compartmental model that dynamically imitates the conditions in the upper part of the GI tract. Fig. 5 shows a schematic illustration of the TIM-1 system displaying four serial compartments representing the GI tract from the stomach through to the small intestine [114]. The temperature is controlled by water jackets, and peristaltic movements is simulated in each compartment by two sections contracting alternately. Pumps and valves ensuring physiologically relevant transit times, as well as addition of gastric, duodenal, jejunal and ileal secretions containing BSs, electrolytes and digestion enzymes (pepsin, ROL, and pancreatin). The bio-accessibility is estimated from filtrates obtained from the jejunal and ileal compartments [114,125]. Similar to the DGM, the TIM-1 model has not been evaluated in relation to predicting the *in vivo* performance of LbDDSs. Yet, the model has shown promise in relation to estimating the *in vivo* performance of LbDDSs, as early studies showed that the model could accurately reproduce *in vivo* data on pH, GI transit, and BS concentrations [82]. Additionally, the model has been used successfully to evaluate DDS effects [112–114], predict food effects [113,126], and predict the *in vivo* performance of an immediate release formulation containing paroxetine hydrochloride hemihydrate [125].

As for all other models, the TIM-1 model has some disadvantages. The model lacks a physiologically relevant absorption step, as the utilized filter system does not mimic active transport, efflux and gut wall metabolism. Presently, in order to predict oral bioavailability instead of bio-accessibility, filtered samples from the jejunal and ileal compartment must be transferred to an intestinal absorption model, *e.g.* a Caco-2 cell monolayer [127]. Due to the complexity of the TIM-1 model, and its many compartments, the performance of the model will depend on the tested drug and DDS, and how these interact with the system [125]. For example, it is a known problem that lipophilic drugs may adsorb to the many plastic surfaces and filters, which can cause a low recovery [1,114]. Compared to testing a lipophilic drug in an aqueous buffer, testing the lipophilic drug in a LbDDS will most likely increase the distribution of the drug in the solvents during transit through the TIM-1 model, which might minimize the adsorption problem. However, as LbDDSs have not been tested in the TIM-1 model it is uncertain whether the adsorption problem will limit the use of the TIM-1 model in relation to evaluating LbDDSs.

5. Current trends for future *in vitro* digestion models

As evident from Section 4, almost all *in vitro* evaluations of LbDDSs, have been conducted using a pH-stat lipolysis model. However, due to the varying IVIVCs, results using this model should be interpreted with care. The fact, that the pH-stat lipolysis model is not *in vivo* predictive for all drugs and all LbDDSs is as such not a fundamental concern, as one model rarely fits all situations. However, as no one, to the authors'

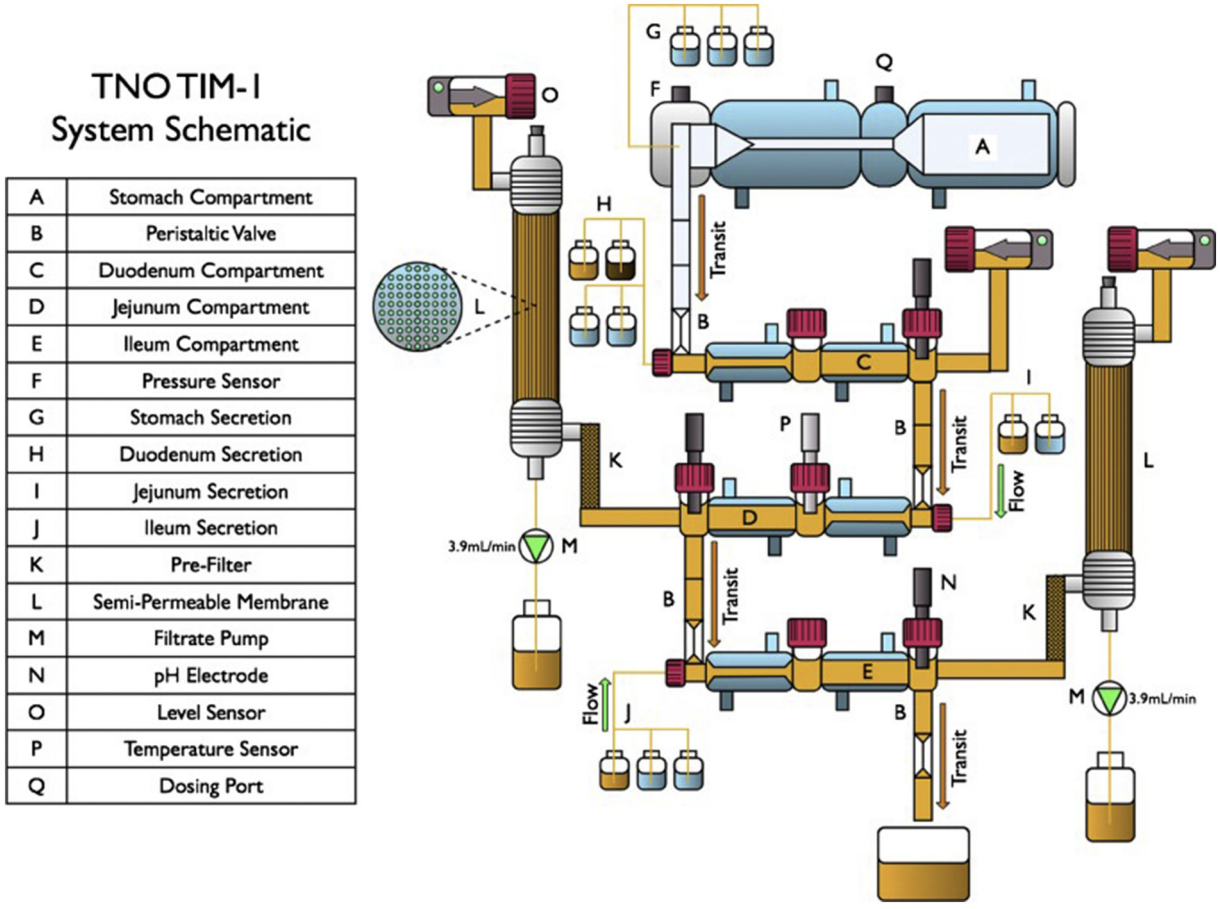


Fig. 5. Schematic drawing of the TIM-1 system. Reprinted from [114] with permission from Springer Nature (2019).

knowledge, has been able to foresee for which drugs or LbDDSs the model is predictive, the pH-stat lipolysis model, as described above, cannot be considered the optimal model for evaluating LbDDSs. Recognizing the shortcomings of the pH-stat lipolysis models, research has been, and is being, conducted towards the development of a predictive *in vitro* digestion model. The following section gives an overview of this research.

5.1. Population- and species specific digestion models

Currently, most *in vitro* digestion models focus on mimicking the conditions of the GI tract of healthy human adults. However, this might be misleading with respect to predicting the oral performance in special populations like the pediatric or geriatric populations, specific patient groups, or when predicting the oral performance in relation to a specific species used in animal studies. While multiple reviews have stated the lack of, and need for, population and species specific digestion models, these are currently scarce [99,128–131]. Of the few existing models, the specific conditions for the simulated population or species, are primarily mimicked by changing the composition of the simulated digestion fluids in the existing pH-stat lipolysis models. Table 3 illustrates key differences in digestion media composition simulating human adults, human neonates and rats, as described in recently published studies [26,85,99,100,132].

5.1.1. Pediatric digestion models

In recent years there has been an increasing regulatory focus on medicines to the pediatric population. According to the newest regulations, the development plan of any new medicinal product must include a Pediatric Investigation Plan (PIP) in the EU and an initial Pediatric Study Plan (iPSP) in the US, including technical aspects and timings of clinical and non-clinical studies [133]. With this increased regulatory focus in the development of DDS for the pediatric population, the focus on predictive *in vitro* models for the pediatric population has also increased [99,130]. Several publications have stressed that the pediatric population cannot be treated as small human adults due to extreme differences in the development of *e.g.* the GI tract [99,128,129]. The physiology of the pediatric GI tract differs not only in fluid volume, but also in pH, BS concentrations, enzyme activities, gastric emptying time, and intestinal transit time, which has been extensively reviewed within the last five years [99,128–130]. Due to the age-dependent development of the GI tract, it is insufficient to use one *in vitro* model setup to depict the whole pediatric population, and therefore the

Table 3
Bile salt concentrations and enzyme activities in suggested compositions for simulated gastric and intestinal digestion media for *in vitro* lipolysis studies simulating human adults [85,132], human neonates and young infants (age 0–2 months, average weight 2.0 kg) [26,99] and rats [100].

State of simulation	Human adult	Human Neonate	Rat
	Fasted	Fed	Fasted
	Gastric medium		
Bile salts	0.08 mM	–	0.08 mM
Phospholipids	0.02 mM	–	0.02 mM
Gastric lipase	50 TBU/mL	17 TBU/mL	–
Pepsin	450 U/mL	126 U/mL	–
pH	2.5	6.4	4.0
	Intestinal medium		
Bile salts	2.95 mM	1 mM	50 mM
Phospholipids	0.26 mM	0.2 mM	3.7 mM
Porcine pancreatic extract	600 USPU/mL	50 TBU/mL	179 USPU/mL
pH	6.5	6.5	6.5

population must be divided into sub-populations. When developing predictive *in vitro* models simulating the conditions of the neonatal and young infant population, it is important to note that this population, in contrast to the adult population, is practically never in a fasted state due to frequent feedings of either breast milk or infant formula [99,128,129]. Furthermore, for this specific sub-population, the pancreatic TG lipase is underdeveloped [72,134], and *in vitro* studies have shown that the pancreatic TG lipase induced digestion is dependent on prior digestion by gastric lipase [135]. Therefore, simulation of both gastric and intestinal conditions is necessary in the development of a neonatal *in vitro* model.

In 2016, Kamstrup et al. suggested an *in vitro* model utilizing the regular pH-stat lipolysis setup with simulated gastric and intestinal media for the neonatal population (age 0–2 months) [99]. Table 3 shows the BS concentration and enzyme activities in the suggested composition of these simulated gastric and intestinal digestion media in the fed state, alongside the composition of the fasted state simulated medium of human adults [26,85,99,132]. The setup of the suggested neonatal *in vitro* lipolysis model is, as previously described, based on the pH-stat lipolysis model using population relevant media and dosing the drug in infant formula in the gastric step, thereby mimicking the frequently fed state of neonates [99]. Klitgaard et al. (2017) evaluated and continued the development of the suggested neonatal *in vitro* lipolysis model by incorporating a physiologically relevant gastric pH drop, and continuous transfer of the gastric contents mimicking gastric emptying (Fig. 3) [26]. Despite the demonstrated advances in biomimicry, the model(s) designed by Kamstrup et al. and Klitgaard et al. have not been validated against *in vivo* data of LbDDSs, and therefore, more studies are needed to evaluate the predictive power of the models. In addition, the neonatal GI digestion models presently share the same problem, as all other gastric digestion models, as they utilize ROL as the gastric lipase [26].

5.1.2. Species specific digestion models

As previously mentioned, *in vitro* models are most commonly designed to mimic the human adult population in order to aid formulation development by predicting *in vivo* behavior, or to elucidate specific mechanisms involved in drug absorption following oral administration. However, as pipeline formulations are always tested *in vivo* in animals prior to clinical studies, and as species differences may lead to wrong predictions of the clinical outcome, it is found relevant to use *in vitro* models designed to mimic the conditions in animals used for *in vivo* studies. With regard to predicting *in vivo* outcome, some studies have suggested that the variable IVIVCs of the pH-stat lipolysis model is caused by the fact, that the results often include interspecies comparison, *i.e.* a comparison between simulated processes occurring in the human GI tract, and the response after an *in vivo* study in *e.g.* rats or dogs [100,131,136]. The physiology of the GI tract of laboratory animals differs from that of humans on many different levels as summarized by *e.g.* Sjögren et al. [131]. While studies have obtained a good correlation between the permeation of drugs in the rat jejunum with that in the human jejunum, the composition of the GI fluids in the rat is quite different from that of humans. As illustrated in Table 3, the rodent GI fluids contain a higher concentration of BSs, and a substantially lower activity of pancreatic lipase [100,131,136].

To avoid interspecies comparisons, Anby et al. studied the *in vitro* lipolysis of three LbDDSs while mimicking the conditions of the rat intestine [136]. The authors utilized *ex vivo* rat pancreatic/biliary fluids determined to have an activity of 194 ± 34 TBU/mL, and a corresponding concentration of pancreatic extract from porcine origin while also considering the GI dilution. *In vitro*, the authors observed a difference in drug distribution when simulating the enzyme activity in rat intestine as compared to that in the human intestine [136]. In a more recent study by Siqueira Jørgensen et al., it was found that simulating the GI fluids and the gastric emptying of the rat rather than the human during *in vitro* pH-stat lipolysis of four LbDDSs, produced much better correlations to *in vivo* PK data obtained in rats [100]. Whereas the authors were unable to relate the data obtained from the human intestinal *in vitro* lipolysis to the *in vivo* data, the modified GI rat lipolysis model with gastric and intestinal media simulating the physiological conditions of the rat intestine (Table 3) and gastric transfer predicted the *in vivo* performance of the four LbDDSs by rank order [100].

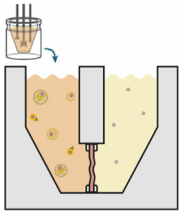
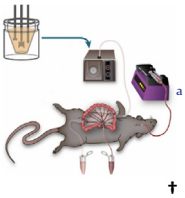
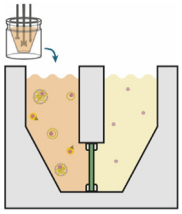
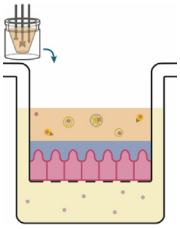
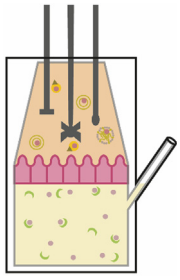
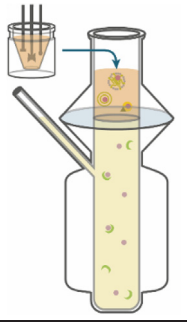
5.2. Combined digestion-permeation models

When *in vitro* studies have failed to predict or reproduce the *in vivo* performance of LbDDSs, the lack of an absorptive sink to take the influence of drug and digestion product removal into account when estimating the performance of LbDDSs, is frequently mentioned among the possible explanations for the missing IVIVC [24,58,104–106]. While several research groups have dedicated time into researching combined models, they have primarily focused on dissolution – permeation models [137–143], and only a few published *in vitro* studies describe the combination of digestion and permeation in the same model [12,27,144–146]. Most of the designed digestion-permeation models utilize the regular pH-stat lipolysis model to simulate digestion, transferring the digestion contents to a separate compartment for the permeation step (Table 4). Therefore, none of these models simulate the simultaneous interaction of digestion and permeation as it occurs *in vivo* [12,27,144]. However, in two recent studies by Keemink et al. and Alskär et al. a newly developed two compartmental model is used to study the lipolysis and permeation of LbDDSs simultaneously (Table 4) [145,146]. Presently, all the models lack proper, extensive validation towards *in vivo* data. Nonetheless, four of the six models were designed and evaluated based on *in vivo* data, while two only focused on validating the integrity of the permeation barrier following incubation with the digestion samples, and thereby the general potential of the experimental setup (Table 4).

Dahan and Hoffman studied the drug distribution of two BCS class II drugs in three LbDDSs in the regular pH-stat lipolysis model, as well as the permeation of the three formulations following 30 min of digestion, across a section of a small intestine from a rat, using side-by-side Ussing chambers [12]. While the authors were able to correlate the amount of drug solubilized in the aqueous phase during *in vitro* lipolysis (for both drugs) to a PK study in rats, they were unable to correlate the results from the *ex vivo* permeation study to the same PK data [12]. Crum et al. were unable to correlate *in vitro* lipolysis data using a regular pH-stat lipolysis model to the *in vivo* situation in their study on three LbDDSs, but when combining *in vitro* lipolysis with *in situ* perfusion in an anaesthetized rat, they successfully predicted the *in vivo* performance observed in previously published studies [28]. In this latter study, the authors perfused the contents of the lipolysis vessel through a section of the rat intestine at a constant rate and quantified the amount of drug in the simulated intestinal fluids before and after passage, as well as the amount of drug in the plasma [28]. While the model produced an IVIVC, and gave valuable mechanical insights into the interplay of drug solubilization, supersaturation, precipitation and absorption of LbDDSs during controlled digestion, the model is too complicated, time consuming, and expensive to be a viable option for the development of LbDDSs, as the authors have also noted [28].

In three more recent studies, *in vitro* lipolysis was combined with *in vitro* permeation, either across a Caco-2 cell monolayer [144], or across artificial bio-mimetic barriers [27,147]. Though several research groups have shown that Caco-2 cell monolayers are incompatible with a long list of LbDDS excipients as well as lipolysis products formed during *in vitro* digestion [148,149], Keemink and Bergström successfully protected the Caco-2 cell monolayer throughout their study using an immobilized lipase, and an additional mucin barrier [144]. The authors transferred the inhibited lipolysis contents following 60 min of lipolysis to a Caco-2 cell monolayer protected by a mucin layer (200 μL of 150 mg/mL mucin). They were unable to utilize the commonly used pancreatic extract of porcine origin as lipase source as the mucin layer

Table 4
Overview of presently designed digestion-permeation models used to evaluate LbDDSs. All models, except Keemink et al., simulate the two processes sequentially using pH-stat lipolysis models followed by permeation studies using different barriers of permeation.

Model design	Dahan and Hoffman [12]	Crum et al. [28]	Bibi et al. [27]	Keemink and Bergström [144]	Keemink et al. [145], Alskär et al. [146]	Klitgaard et al. [147]
						
Setup for permeation	Ussing chambers	*In situ* rat perfusion	Ussing chambers	Transwell system	Two compartmental simultaneous setup	Franz diffusion cell
Transfer of lipolysis content	Following 30 min of controlled lipolysis	Continuous transfer by peristaltic pump	Following 10 min of controlled lipolysis	Following 60 min of controlled lipolysis	Simultaneous	Following 0, 15, 30, and 60 min of controlled lipolysis
Permeation barrier	Rat jejunum (*ex vivo*)	Rat jejunum (*in situ*)	PermeaPad®	Caco-2 monolayer	Caco-2 monolayer	PermeaPad®
Acceptor medium	Modified Ringer buffer	Mesenteric vein	FaSSIF-V2-Blank buffer	HBSS	HBSS with 4% (w/v) BSA	PBS with 4% (w/v) BSA
IVIVC from lipolysis	Yes	No	Not evaluated	Not evaluated	No	No
IVIVC from combined setup	No	Yes	Not evaluated	Not evaluated	Yes	Yes

Abbreviations: BSA: Bovine serum albumin, HBSS: Hank's balanced salt solution, PBS: Phosphate buffered saline.
[a] Modified and reprinted with permission from Springer Nature (2019).

was insufficient to protect the monolayer against the cytotoxic effect of this lipase. As a substitute, the authors utilized an immobilized microbial lipase as source for the digestive enzyme [144]. Consequently, the digestion profiles and extent of digestion were different compared to those obtained using the pancreatic extract of porcine origin. Whether this has an influence on the predictability of the model is currently unknown, as the model has yet to be validated against *in vivo* data. Furthermore, the thickness of the protective mucin barrier far exceeded the physiological relevant range as reported by Ensign et al. [150]. Whether this has an influence on the predictability of the model is for future studies to conclude. In the studies by Bibi et al., and Klitgaard et al. (in prep.), samples from a regular pH-stat lipolysis digestion experiment of LbDDS, were transferred to an artificial PL-based biomimetic membrane (PermeaPad®) in side-by-side Ussing chambers, or Franz diffusion cells, respectively [27,147]. Similar to Keemink and Bergström, Bibi et al. focused primarily on evaluating the integrity of the PermeaPad® barrier in combination with the *in vitro* digestion samples of a cinnarizine containing LbDDS (self-nano-emulsifying drug delivery system, SNEDDS) (Table 4). Initiating the digestion in the pH-stat lipolysis model, they transferred the uninhibited digestion samples after 10 min of controlled digestion to a side-by-side Ussing chamber equipped with the PermeaPad® barrier and FaSSIF-V2-Blank buffer in the acceptor compartment and studied the permeation of cinnarizine and calcein (used as a permeation marker molecule) [27]. The authors concluded that the PermeaPad® barrier maintained integrity during exposure to the digestive conditions, and with this, that the model was a promising tool for screening LbDDSs. However, they did not relate the findings to an *in vivo* study or validate the model in any other way [27]. Klitgaard et al. (in prep.) used the same principle for the experimental setup as Bibi et al., and transferred uninhibited samples from the lipolysis of four formulation approaches using SNEDDSs to Franz diffusion cells equipped with PermeaPad® barriers (Table 4). To ensure sink conditions, the acceptor medium consisted of phosphate buffered saline at pH 7.4 with 4% (*w/v*) bovine serum albumin (BSA). Like Bibi et al., the authors concluded that the setup was stable under the chosen conditions and managed to maintain a steady state flux throughout the study. Furthermore, Klitgaard et al. were able to correlate the results from their setup to previously published *in vivo* data of the same formulations [147]. While this setup is promising, more studies are needed to validate the model.

In a recent study by Keemink et al., the authors utilized their experience with combining lipolysis with permeation across a Caco-2 monolayer to develop a new two-compartmental setup to study the lipolysis-permeation interplay simultaneously [145]. The new setup consisted of a cone-shaped lipolysis vessel as a top donor compartment separated from the lower acceptor compartment by a Caco-2 monolayer (Table 4). Similar to the regular pH-stat *in vitro* model, the upper compartment had stirring and pH control, and the lower compartment was structured similarly to a Franz diffusion cell, although larger. As in their previous studies, immobilized microbial lipase was used rather than the porcine pancreatic extract, however, in this newest study, the mucin barrier was not used to protect the Caco-2 cell monolayer. The authors tested six LbDDSs in the new setup; three formulations (F1-F3) for method development and another three (F4-F6) for comparison to previously published *in vivo* data. The authors found that the formulations ranked differently when adding the permeation step as compared to running the regular pH-stat *in vitro* lipolysis with the immobilized lipase. Interestingly, when testing the same formulations in the pH-stat lipolysis model with the porcine pancreatic extract rather than the immobilized lipase, the authors obtained the same rank ordering as was observed in the combined lipolysis-permeation study of the formulations. However, as these formulations were not tested *in vivo,* no *IVIVC* was obtained. When testing F4-F6 in the new setup, the author accurately predicted the oral performance of the formulations by rank order [145]. Alskär et al. used the same two-compartmental, simultaneous setup as Keemink et al. to test another three LbDDSs and compared them to the *in vivo* performance after oral administration to dogs [146]. In this study as well, the authors managed to predict the oral performance of the tested LbDDSs by rank order. Based on these results, the simultaneous lipolysis-permeation model designed by

Keemink et al. shows promise, but still needs to be tested more extensively for validation.

The recent studies of combined *in vitro* lipolysis and permeation show an interesting and promising development in moving towards the establishment of predictive *in vitro* digestion models. The currently proposed models have different possibilities and limitations. While artificial barriers mimic the intestinal wall to a lesser degree as compared to Caco-2 cells, the PermeaPad® barrier was not only able to maintain stability when exposed to common conditions during *in vitro* lipolysis, but also differentiate between the tested formulations to the degree of obtaining an IVIVC, which was not possible using the standard pH-stat lipolysis model [147]. Furthermore, the work on the simultaneous, two-compartmental model opens up for new possibilities to obtain IVIVCs by combining the Caco-2 monolayer with immobilized microbial lipase. Future extensive studies are still needed to evaluate the potential of any of the combined digestion-permeation models. Furthermore, it is important to note, that while the combined models described by Keemink et al. [144–146], Bibi et al. [27], and Klitgaard et al. [147], are simpler and less time consuming, as compared to the lipolysis – rat perfusion model described by Crum et al., they are still relatively complex and currently unfit for larger scale screening purposes of LbDDSs.

6. Conclusions and future perspectives

In vitro digestion models have been widely used to study the GI behavior of LbDDSs. Most of these models are based on the pH-stat lipolysis model, simulating the enzymatic digestion processes occurring in the human (stomach and) small intestine. Despite a number of studies showing IVIVCs between the amount of drug solubilized in the aqueous phase during *in vitro* lipolysis in the pH-stat lipolysis model and preclinical PK data, an increased number of studies have highlighted that the model is not always predictive. While the DGM, and the TIM-1 broaden the range of *in vitro* digestion models available to forecast the *in vivo* performance of LbDDSs, and are simulating both the mechanical and the enzymatic digestion processes, limitation in terms of mimicking intestinal drug permeation and practical issues as their low throughput limit their use.

Presently, no single *in vitro* digestion model exists which is able to fully and consistency predict the *in vivo* performance of LbDDSs. However, recent research has shown the potential of combined digestion-permeation models as well as patient and species specific digestion models. As described above, several digestion-permeation models are currently being evaluated, and the authors are expecting that a preferred model, predicting *in vivo* absorption from LbDDSs will soon be identified. Furthermore, the authors believe that the patient and species specific digestion models will gain more attention in the future, based on the general focus on personalized medicine. Tailoring the *in vitro* models to mimic special populations or species will facilitate this development.

All in all, many activities are currently ongoing to improve the *in vivo* predictability of the existing *in vitro* digestion models. Within the next few years, these efforts are expected to materialize into better models which are able to predict the *in vivo* performance of LbDDSs.

Declaration of interest

The authors declare no conflicts of interest.

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