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Review Article

Theme: Develop Enabling Technologies for Delivering Poorly Water Soluble Drugs: Current Status and Future Perspectives
Guest Editors: Ping Gao and Lawrence Yu

Characterising Lipid Lipolysis and Its Implication in Lipid-Based Formulation Development

Nicky Thomas,[1] René Holm,[2] Thomas Rades,[1,3] and Anette Müllertz[3,4,5]

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Abstract. Facing the increasing number of poorly water-soluble drugs, pharmaceutical scientists are required to break new grounds for the delivery of these pharmaceutically problematic drugs. Lipid-based drug delivery systems (LBDDS) have received increased interest as a novel drug delivery platform during the last decades and several successfully marketed products have shown the potential for LBDDS. However, there exists a discrepancy between the clear need for innovative delivery forms and their rational design. In the case of LBDDS, this can be attributed to the complexity of LBDDS after administration. Unlike conventional formulations, LBDDS are susceptible to digestion in the gastrointestinal tract, the interplay of delivery system, drug and physiology ultimately effecting drug disposition. *In vitro* lipolysis has become an important technique to mimic the enzymatic degradation. For the better understanding of how LBDDS promote drug delivery, *in vitro* lipolysis requires advanced characterisation methods. In this review, the physiological background of lipid digestion is followed by a thorough summary of the techniques that are currently used to characterise *in vitro* lipolysis. It would be desirable that the increasing knowledge about LBDDS will foster their rationale development thereby increasing their broader application.

KEY WORDS: *in vitro* digestion; *in vitro* lipolysis models; lipid-based drug delivery systems; poorly soluble drugs; self-nanoemulsifying drug delivery systems (SNEDDS).

INTRODUCTION

It has long been observed that the intake of food, notably lipids, can have profound effects on the absorption and bioavailability of drugs (1). In particular, the bioavailability of poorly water-soluble, lipophilic drugs has been shown to benefit from the concomitant ingestion of lipids, sparking the interest in the use of lipids as potential drug delivery systems (2,3). As a result some compounds formulated as lipid-based drug delivery systems (LBDDS) have entered the market successfully (4). However, there appears some reluctance in the broader utilisation of LBDDS, indicated by the still-limited number of products commercially available (4,5). This might be attributed to the intricate interplay of LBDDS and the digestive system. After administration, LBDDS are processed in a complex physiological sequence which is not completely understood. It is this inherent alteration of the drug carrier during its transit through the gastrointestinal tract (GIT) which distinguishes LBDDS from conventional formulations such as tablets. Consequently this attribute asks for protocols capable of assessing and predicting the quality and performance of LBDDS. However, standardised protocols have not been established yet, which can be considered a major obstacle for the broader use of these delivery systems. This review sets out to summarise the recent developments in the characterisation of LBDDS. In the following sections, an overview of the physiological processes in the GIT necessary for the understanding of the fate of LBDDS after oral administration, is presented. Thereafter, the evolution of meaningful *in vitro* methods necessary for the development and rational testing of LBDDS is highlighted.

LIPID-BASED DRUG DELIVERY

LBDDS are a heterogeneous group of delivery systems comprising solutions of oil, emulsions, suspensions, liposomes and self-emulsifying drug delivery systems (SEDDS) (3,5). The latter can be considered the most complex formulations consisting of oil, surfactant, co-surfactant, cosolvent, and drug (3,4). Upon contact with aqueous medium these isotropic

[1] School of Pharmacy, University of Otago, 9054 Dunedin, New Zealand.
[2] Preformulation, H.Lundbeck A/S, Ottiliavej 9, 2500 Valby, Denmark.
[3] Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, 2100 Copenhagen, Denmark.
[4] Bioneer:FARMA, Faculty of Health and Medical Sciences, University of Copenhagen, 2100 Copenhagen, Denmark.
[5] To whom correspondence should be addressed. (e-mail: amu@farma.ku.dk)

preconcentrates spontaneously generate coarse emulsions, or fine nano-emulsions, referred to as self-nanoemulsifying drug delivery systems (SNEDDS). In the literature, the term SNEDDS is often used in parallel with the term self-microemulsifying drug delivery system (SMEDDS) without further distinction of the true nature of the resulting dispersion. SMEDDS are defined as thermodynamically stable systems whereas SNEDDS are kinetically stable. According to a recent review by Anton, most of the studies that describe SMEDDS are, in fact SNEDDS (6). Therefore, throughout this work the term SNEDDS is used and reference is made to the terminology of other authors where applicable. SNEDDS and SMEDDS have gained considerable attention since the large surface area associated with the fine dispersion of these systems (droplet size, <<100 nm) is believed to foster rapid digestion followed by a more reproducible drug release and absorption compared with non-dispersing LBDDS (2).

Similar to food-derived lipids, the digestive system metabolises LBDDS in a complex interplay with the GIT fluids resulting in the solubilisation and distribution of the co-administered drug between mixed micelles or other colloidal phases potentially facilitating drug absorption. Other mechanisms than enhanced solubilisation might also contribute to improved bioavailability: it is well documented that the lymphatic uptake of very lipophilic compounds can be an alternative route of drug absorption which can be stimulated by long-chain lipids commonly used in LBDDS (7–10). Furthermore, some excipients have been shown to impede the activity of P-glycoproteins and intestinal cytochrome P450 and to enhance the permeability of the intestinal epithelium potentially giving rise to improved absorption (11,12). Finally, gastric emptying can be protracted in response to lipids allowing more time for drug dissolution (13).

LIPID DIGESTION

Gastric Digestion

Lipids are an essential part of the nutrition constituting of up to 170 g of the daily diet in contemporary Europe (14,15). Notwithstanding the problems associated with such a high-energy intake, the physiology of the body is well equipped for the assimilation of lipids, starting in the stomach. Here, lingual (in some species (16)) and gastric lipases, secreted by the salivary glands and the gastric mucosa, respectively, are the first enzymes starting hydrolysis of the lipids (17). Both enzymes have similar preferences regarding the substrate: lipids consisting of medium-chain triglycerides are hydrolysed faster compared with long-chain triglycerides, both reactions producing diglycerides, monoglycerides and (partially unionised) fatty acids. The surface activity of the digestion products together with the dietary phospholipids and the powerful shear forces of the stomach promote the first crude emulsification of the lipids (17). After emulsification in the stomach, the lipid droplets are propelled into the duodenum by the contractions of the pylorus for further processing. On a quantitative basis, the gastric lipolysis step accounts for up to approximately 10–25% of the total lipolysis, whereas the rest of the lipids are hydrolysed by pancreatic enzymes (18,19).

Intestinal Digestion

The arrival of the acidic lipid emulsion in the duodenum triggers several events crucial for the effective and complete hydrolysis of the remaining lipids (17). The pancreas secretes a mixture of fluids containing enzymes of which pancreatic lipase and co-lipase are most important. The secretion of bicarbonate elevates the pH to approximately 6–8, reflecting the pH optimum for pancreatic lipase (20). Pancreatic lipase selectively hydrolyses triglycerides at the Sn1 and Sn3 position, producing 1 mol 2-monoglycerides and 2 mol fatty acids for each mole triglyceride. Similar to gastric lipase the efficiency of pancreatic lipase towards hydrolysis of medium-chain triglycerides is enhanced compared with long-chain triglycerides (20). However, the hydrolytic activity of pancreatic lipase highly depends on a complex interplay with co-lipase, bile acids and calcium.

One of the prominent properties of pancreatic lipase is that the enzyme is active only at the oil/water interface (21). Therefore, its activity is highly influenced by any processes affecting the surface of the substrate. This is particularly important during the progress of lipolysis where the surface of the substrate accumulates amphiphilic digestion products, including fatty acids and 2-monoglycerides that form liquid crystalline and 'viscous isotropic' phases (15,22,23). The accumulation of these compounds restricts the access of pancreatic lipase to the oil/water interface. It has been shown that bile acids can displace these amphiphiles from the substrate interface. However, bile acids can also inhibit pancreatic lipase (24). Co-lipase binds to pancreatic lipase in a 1:1 complex reconstituting the activity of pancreatic lipase and serving as an anchor for pancreatic lipase at the substrate interphase (20,25). Furthermore, co-lipase stabilises pancreatic lipase in an 'open-lid' conformation (26). The lid comprises a surface loop covering the catalytic centre in the inactive conformation of the enzyme, thereby restricting access to its catalytic centre (21). Stabilisation of the open lid, therefore, increases the likelihood of substrate hydrolysis (18,27).

For an optimal activity of the pancreatic lipase/co-lipase complex, the presence of bile salts is required (28). The most important bile acids in humans are cholic acid, deoxycholic acid and chenodeoxycholic acid. The conjugation with taurine and glycine renders the bile acids more surface active. This facilitates the displacement of digestion products and formulation-derived surfactants from the interface. The accumulation of these compounds would result in the detachment of the pancreatic lipase from the interface thereby preventing further lipolysis. Furthermore, *in vivo,* bile salts aid in the removal of digestion products from the interface by solubilisation into mixed micelles facilitating the transport across the unstirred water layer and the intestinal mucus into the enterocyte by a mechanism still unknown. Medium-chain fatty acids diffuse directly across the enterocyte accessing the systemic circulation *via* the portal vein, whereas long-chain fatty acids require re-esterification to triglycerides (29,30). This is accomplished at the endoplasmic reticulum of the enterocyte, followed by the incorporation of the newly synthesised triglycerides into lipoproteins and their subsequent release into the lymphatic system.

Calcium ions have been described as an additional factor regulating lipolysis *in vivo* (15,22,31). The calcium concentration changes considerably from approximately 3–4 mM in the fasted to approximately 15 mM in the fed state (32). An increase in the calcium concentration has been associated with a decline in the lag phase commonly preceding maximum lipase activity (33). It is assumed that calcium improves the penetration of the pancreatic lipase into the substrate surface by the screening of negative charges from the surface decreasing the electrostatic repulsion of the enzyme (33). Other authors suggested a highly enzymatically active complex of enzyme, phospholipid, mixed micelle and calcium (34). Furthermore, calcium is known to generate liquid crystals with bile salts (22). Free fatty acids are solubilised in this liquid crystalline phase followed by the absorption of the fatty acids by the intestinal epithelium. This mechanism is important as it presents the removal of digestion products from the substrate surface.

The preceding discussion illustrates the dynamics and complexity of lipid digestion. Generally, similar considerations apply to the digestion of lipids originating from LBDDS although the lipid amounts are generally smaller compared with those deriving from food products. Furthermore, excipients (*e.g.* surfactants) that are not present in food, might be used in LBDDS. Therefore, LBDDS pose particular challenges in regard to their quality assessment and *a priori* prediction of their performance.

DISPERSION TESTING OF LBDDS

Standardised techniques such as compendial dissolutions studies used for the quality assessment of conventional formulations do not apply to LBDDS as the co-administered drug is commonly presented in solution. However, the same equipment used for compendial dissolution testing (*e.g.* USP paddle 2) has been used frequently to study the dispersion characteristics of some LBDDS (35–37). With regard to SNEDDS which have attracted most attention amongst LBDDS, compendial media have been used mainly for the assessment of the dispersion characteristics, *i.e.* the dispersion time, droplet size or zeta potential of dispersed formulations (35,38,39). Interestingly, despite the commonly employed dispersion assessment during the development of SNEDDS, the target values for dispersion have not been defined. The rationale behind the assessment of droplet size is the observation that an increased surface area, as generated by SNEDDS, allows rapid and reliable digestion by lipases facilitating drug partitioning in the solubilising phases (40). In this context two products of the immunosuppressant drug cyclosporine A may be mentioned: Neoral®, forming a microemulsion upon dispersion, and its predecessor Sandimmun® which produces a coarse emulsion. Clinically, Neoral® has shown improved bioavailability and dose linearity compared with the coarse emulsion (40,41). Although this finding stimulated the development of similar SNEDDS, the question whether dispersion alone can determine the performance of LBDDS, has not been sufficiently answered yet. It should be noted that Neoral® in addition contains excipients which have been shown to interact with P-glycoprotein and cytochrome P450. Therefore, factors other than dispersion related, cannot be ruled out (42).

Furthermore, dispersion cannot be assessed for non-dispersing LBDDS such as for formulations based on pure triglycerides. In this case the drug transfer from the lipid into the test medium rather than the dispersion characteristics might be more relevant to assess, which requires the choice of an appropriate test medium. During the last decades, the thorough physicochemical characterisation of human gastrointestinal fluids allowed the development of biorelevant media closer matching the *in vivo* conditions compared with the rather simple compendial media (43–45). The proposed biorelevant media contain physiological relevant amounts of buffer, lecithin and bile salts mimicking either the fasted or fed state (Table I) and have been subject of recent reviews (46,47).

Despite the progress in the development of dissolution media the major drawback of most dissolution media is the fact that they are rather static systems that have not been designed to reflect the dynamic changes in the GIT occurring after ingestion of a meal. In contrast, lipid digestion is a highly dynamic process during which amphiphilic species are generated, colloidal structures appear and change over time, which will in turn affect drug distribution and, finally, drug absorption. In the case of LBDDS that are often susceptible to digestion before they deliver the co-administered drug, a dynamic model appears therefore more appropriate.

IN VITRO LIPOLYSIS

In vitro lipolysis is typically carried out in a thermo-controlled reaction vessel containing digestion medium representative of either the fasted or the fed state (Fig. 1).

Although the composition varies between different laboratories (Table II), the digestion medium commonly comprises an aqueous buffer solution, supplemented by bile salts, phospholipids and NaCl (48). The concentrations of these compounds are defined by the physiological conditions prevailing in the fasted or fed state. The pH of the medium is monitored by a pH-sensitive electrode connected to a computer-controlled pH-stat device capable of maintaining a predefined pH value *via* titration with NaOH. Upon addition of pancreatic lipase and co-lipase the hydrolysis of triglycerides and other digestible excipients present in the formulations is initiated. As a result of the enzymatic hydrolysis, free fatty acids are released into the digestion medium leading to a

Table I. Composition of the Fasted- and Fed-State Simulated Intestinal Fluids Proposed for Biorelevant Dissolution Testing (FaSSIF-V2 and FeSSIF-V2)

Composition/property	FaSSIF-V2	FeSSIF-V2
Sodium taurocholate (mM)	3	10
Lecithin (mM)	0.2	2
Maleic acid (mM)	19.12	55.02
Sodium chloride (mM)	68.62	125.5
Sodium hydroxide	34.8	81.65
Glyceryl monooleate (mM)	–	5
Sodium oleate	–	0.8
Osmolality (mOsm kg^{-1})	180±10	390±10
Buffer capacity (mmol $l^{-1}\Delta pH$)	10	25
pH	6.5	5.8

Adapted from Jantratid *et al.* (49)

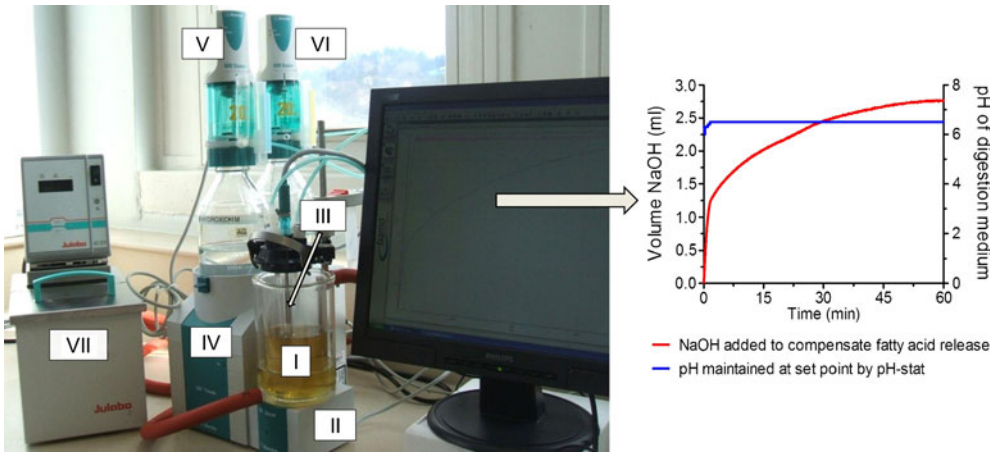

Fig. 1. The *left panel* shows the typical lipolysis set up. The thermo-jacketed reaction vessel (*I*) contains the digestion medium and is stirred constantly by the magnetic stirrer (*II*). The pH-electrode (*III*) measures the pH and temperature of the lipolysis medium. The pH-stat unit (*IV*) regulates the dispensing of sodium chloride (V) and calcium chloride (*VI*). The water bath (*VII*) maintains the temperature of the lipolysis medium at 37°C. The *right panel* depicts the typical profile of a lipolysis experiment where the pH of the lipolysis medium is maintained constant (*blue line*). The red line represents the cumulative consumption of NaOH to titrate the liberated fatty acids during *in vitro* lipolysis

drop in the pH. The change in the pH is immediately compensated by the titration with NaOH. In order to reduce the experimental error and to obtain reproducible results, the concentration of the NaOH solution needs to be adequate to compensate for the release of fatty acids expected to be generated from the amount of lipids during lipolysis. In case of a NaOH concentration chosen too low relative to the amount of substrate, this will result in the dilution of the digestion medium. Since the solubilisation capacity for a drug is a function of the bile salt concentration in the medium,

Table II. Examples of *In Vitro* Lipolysis Conditions

Volume (ml)	Duration (min)	pH	Buffer	Lipase	Bile/ phospholipid	Calcium	Substrate	References
9	12	6.5	Tris-maleate (2 mM)	Pancreatin (24 TBU/ml)	NaTDC (8 mM)	10 mM	0.45 ml TG emulsion	(50)
5		8.5	Tris-maleate (50 mM)	Porcine pancreas (168–280 TBU/ml)	NaTDC (0–30 mM/ lecithin)	0–30 mM	0–5% (*w*/*v*) of TG emulsion (10%) emulsified with PL	(34)
10		6–9	Tris-maleate (2 mM)	Purified from human pancreatic fluid (1–8 TBU/ml)	NaTC NaTDC or NaCDC (6 mM)	10 mM	0.5 ml olive oil emulsified with gum Arabic	(24)
40	30	6.8–7.4	Tris-maleate (50 mM)	Porcine pancreatin ((8× USP) 1,000 IU/ml)	NaTC (5 mM L-α-PC)	5 mM	0.2–1 g TG	(51,52)
37.5	20–120	7.0	Phosphate (5 mM)	Porcine pancreas type II (L3126, Sigma; (100–400 U/mg, 2.4 mg/ml)	Porcine bile extract (20 mM)	10 mM	0.5% TG emulsion, different emulsifiers,1%	(53)
300	40–90	6.5	Tris-maleate (2 mM)	Porcine pancreatin (3× USP; 300–800 USP units/ml)	Porcine bile extract (5–30 mM)/1–5 mM PC	Continuous addition (0.045–0.181 mmol/min)	15 mM -30 mM TG,1-3 g SNEDDS,	(37,48,54, 55,56)
10–40	30–60	7.5	Tris-maleate (50 mM)	Porcine pancreatin (8× USP; 1,000 TBU/ml)	NaTDC (5–20 mM)/ 1.25–5 mM lecithin (60% PC)	5 mM	1 g SEDDS , TG	(57,58)
100	40	6.5	Tris-maleate (50 mM)	Pancreatin extract (8 TBU/mg)	Bile salts (5 mM)/ 1.25 mM lecithin	5 mM	1 g SEDDS	(59)
20	35	7.5	Tris-maleate 50 mM	Porcine pancreatic lipase 40,000 IU/g 4,000 TBU/ml	NaTDC 5 mM/1.25 mM lecithin (92% PC)	5 mM	0.21 g TG, sub-microemulsion	(60)

FFA free fatty acids, *NaTC* sodium taurocholate, *NaTDC* sodium deoxytaurocholate, *NaCDC* sodium chenodeoxycholate, *PC* phosphatidylcholine, *PL* phospholipid, *TBU* Tributyrin unit (1 TBU equals the amount of enzyme that can liberate 1 mol of fatty acid from tributyrin/min) (69)

dilution will lead to an underestimation of drug solubilisation. On the other hand, too-concentrated NaOH solutions bear the risk of over titration and the erroneous reading of small volumes of the titrant.

Due to the stoichiometry of the hydrolysis reaction, 2 mol NaOH are needed for the hydrolysis of 1 mol triglyceride. Complete hydrolysis of the 2-monoglycerides to glycerol and a third mole fatty acid can be mediated either by isomerisation of 2-monoglycerides to 1-monoglycerides in alkaline medium (61) or by hydrolysis directly at the Sn2 position by the less specific carboxyl ester hydrolase (cholesterol esterase) (62). As the velocity of 2-monoglyceride absorption by the intestine is faster compared with the isomerisation reaction (17), the relative generation of glycerol during triglyceride hydrolysis is rather limited, accounting for approximately 22% of the overall digestion products (20).

During *in vitro* lipolysis samples are withdrawn, followed by the addition of a lipase inhibitor (commonly 4-bromobenzeneboronic acid) to stop further lipase activity within the sample. Thereafter, the samples are analysed either directly or, more frequently, after a centrifugation or ultracentrifugation step separating the sample in two or more phases depending on the sampling time point and on the composition of the formulation (Fig. 2) (63–65). At the beginning of *in vitro* lipolysis, when more undigested lipids are present, it is more likely to obtain an oil phase as the top layer. However, the presence of a lipid layer also depends on the initial composition of the investigated LBDDS. SNEDDS and SMEDDS commonly produce no lipid layer due to their fine dispersion and reduced lipid content. In contrast, non-dispersing LBDDS (*e.g.* pure soy bean oil) generate a lipid layer on top of the lipolysis medium. Provided the lipids employed are digestible by the lipases the oil layer diminishes with the progress of *in vitro* lipolysis. At the same time, the amount of pellet is increasing due to the precipitation of fatty acid as calcium soaps (see below).

The physiological pH range of the intestine (pH 6.1–7.3 in the fasted and pH 5–6.6 in the fed state, respectively) (47) includes the pH optimum of the pancreatic lipase/co-lipase complex (reported between pH 6 and 9) (20,31). Consequently, *in vitro* lipolysis should be conducted closely to these values to reflect the *in vivo* conditions. From an experimental point of view however, the chosen pH needs to be high enough to present the fatty acids in a titratable ionised form (24). The apparent pK_a of long-chain fatty acids in lipolysis medium is lower compared with that in water (*e.g.* approximately pK_a 6.5 compared with pK_a 8 for oleic acid) probably due to the presence of mixed micelles and Ca^{2+} ions (48,66). Thus, choosing a physiological pH between 6 and 7, the free fatty acids can—at least to some extent—be titrated (48,54). It has been recommended to increase the pH of the lipolysis medium after the actual lipolysis experiment to values above the pK_a of the fatty acids (*e.g.* to pH 9) to titrate all the fatty acids liberated during *in vitro* lipolysis which might have been obscured at lower pH (67).

The titration of fatty acids with NaOH is an indirect and unspecific method to monitor lipid hydrolysis. It is noteworthy that a drop in the pH (resulting in an increased NaOH consumption) can partly originate from the absorption of CO_2 by the lipolysis medium (68). Other sources of fatty acids might derive from the degradation of proteins, the hydrolysis of impurities present in the crude extracts of bile and pancreas, and the hydrolysis of phospholipids present in the lipolysis medium (63,68). In order to compensate for this 'background lipolysis', blank experiments are carried out that involve the 'digestion' of pure (*i.e.* formulation free) lipolysis medium and subsequent subtraction of the recorded NaOH volumes from the formulation-containing lipolysis experiments (55,57).

Since *in vitro* lipolysis generates fatty acids that potentially inhibit further enzymatic activity of the pancreatic lipase by accumulation at the lipid surface, the end products of *in vitro* lipolysis have to be removed. *In vivo* this removal is accomplished by the absorption of fatty acids by the intestinal wall. In lieu of this physiological mechanism, the addition of Ca^{2+}-ions precipitates the fatty acids as calcium soaps during *in vitro* lipolysis (22). For the addition of Ca^{2+}-ions, two main strategies are followed: either the addition as a bolus at the start of lipolysis (68,51,69), or the continuous Ca^{2+}-addition by a peristaltic pump, also referred to as the 'dynamic' *in vitro*

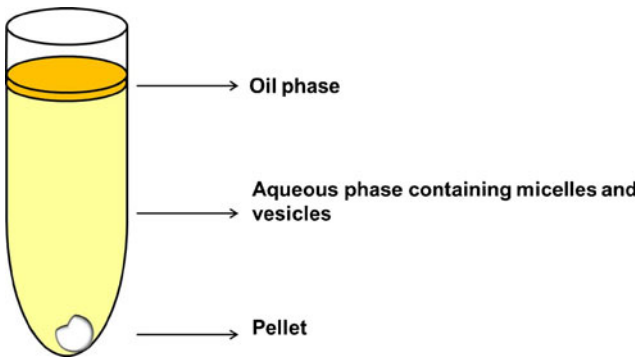

Fig. 2. Schematic of the lipolysis medium after ultracentrifugation. The lipolysis medium separated into at least two phases: the pellet, containing mostly calcium soaps of fatty acids and precipitated drug; the aqueous phase containing the drug solubilised in mixed micelles and vesicles; the oil phase containing undigested lipids. Redrawn and adapted from reference (70)

lipolysis model (48,54). The bolus Ca^{2+}-addition (commonly 5 mM) results in a very fast hydrolysis rate with lipolysis being almost complete within the initial 10 min (57) while the continuous Ca^{2+}-addition (*e.g.* 0.181 mmol/min) allows a controlled lipolysis rate (48,54).

Another difference between the currently employed digestion models arises from the source of bile acids. While the concentration of bile is similar (5–30 mM) throughout the employed lipolysis models, some groups utilise pure taurocholic acid (51,52,71) or taurodeoxycholic acid (57,58,72), whereas others employ a crude (porcine) bile extract (47,73,56). The utilisation of pure bile acids renders the composition of the lipolysis medium more reproducible compared with the more variable crude extract, but lacks the complex composition of human bile (63). Recent studies have shown that the bile acid concentration rather than the structure of the bile acids affects the drug solubilisation in artificial intestinal fluids suggesting that the use of a single bile acid could reflect the physiological situation (74,75). However, these studies were not carried out during *in vitro* lipolysis where the phenomena at the lipid/water interface have been shown to be sensitive to the different structures and hydrophobicities of a range of bile acids potentially interfering with the lipase activity (33). Therefore, compared with pure bile salts, crude porcine bile extract might provide some advantage, including economic considerations.

The ultimate objective of the *in vitro* lipolysis model is to predict the *in vivo* performance of LBDDS *a priori* from the corresponding *in vitro* design parameters, hence to achieve *in vitro/in vivo* correlations. So far, the development of LBDDS has been based on a trial and error approach rather than on rational design. Probably the two most important reasons for this are the heterogeneity of LBDDS, comprising of up to five and more excipients (4) and the intricate interactions of the formulation components with the complex endogenous digestion apparatus. Although the knowledge of the physicochemical processes underlying lipolysis has grown during the last decades, one of the most probing questions remains yet unanswered: what are the determinants of successful and predictable drug absorption from LBDDS and their digestion products? Several *in vivo* studies have been able to demonstrate rank order *in vitro/in vivo* correlations (52,76,77). However, *in vivo* studies are time consuming and costly. Therefore, it would be desirable to apply predictable *in vitro* models to the design and assessment of LBDDS.

The currently employed lipolysis models emphasise the intestinal digestion while giving only little attention to the pre-intestinal lipolysis. As mentioned above the activity of gastric lipase can account for approximately 10–25% of the total lipid digestion and the enzyme remains active in the intestine (20). Since the products of gastric lipolysis are surface active, facilitating the emulsification of the lipid before entering the intestine for further lipolysis (17), the gastric lipolysis step might be of greater importance for the assessment of LBDDS than previously assumed. The impact of gastric lipase activity is confirmed further by the observation of sustained lipid absorption in patients suffering from cystic fibrosis despite the incompetence of the patient's pancreas to secrete lipase (78,79). Further studies will help elucidate the importance of pre-pancreatic lipolysis as part of *in vitro* digestion models.

PERMEABILITY STUDIES WITH LBDDS

So far none of the current *in vitro* lipolysis models has routinely accommodated an absorption model following the *in vitro* digestion step. The literature offers only very limited information regarding *in vitro* drug permeability and absorption following the digestion of LBDDS. The effects of some formulation parameters (oil structure, surfactant HLB, lipid/surfactant ratio) on the intestinal permeability and drug release kinetics from LBDDS were investigated by Buyukozturk *et al.* (80) and the effect of SMEDDS on tight junctions was evaluated by Sha *et al.* (81). Using Caco-2 cell line monolayers the studies revealed useful considerations for the design of LBDDS, including the potential toxicity of these formulations towards Caco2-cell lines, as evident from the observed disruption of the tight junctions. However, in these studies the investigated formulations were diluted with rather simple buffer solutions devoid of bile salts and lipases, making the extrapolation to the complex intestinal environment difficult. In a study by Dahan *et al.* (52), the *in vitro* lipolysis model was able to predict the bioavailability of dexamethason and griseofulvin. On the other hand, when the intestinal permeability of the drugs was assessed from samples obtained during *in vitro* lipolysis in an Ussing chamber, the *ex vivo* model failed to predict the performance seen *in vivo* and during *in vitro* lipolysis (52). It appears that suitable *in vitro* models capable of withstanding the harsh conditions of representative intestinal conditions have yet to be established. This will ultimately provide a tool for the assessment of LBDDS needed to create a guide for a more rational design of LBDDS.

CHARACTERISATION TECHNIQUES DURING *IN VITRO* LIPOLYSIS

The rationale of LBDDS is to increase the absorption of poorly water-soluble, lipophilic compounds by improved solubilisation mediated by the interaction of the drug with endogenous and exogenous surfactants, lipids and their digestion products. Although research in lipid digestion is well established (22,61,66), the interest of pharmaceutical scientists in this area is relatively new. The solubility of a drug is intrinsically linked to the fate of the formulation and drug distribution is dictated by the type of colloidal phases generated during digestion (56,82–85). The characterisation techniques (Table III) have been focussed either on the evolution of the colloidal digestion products or on the solubility and distribution of the co-administered drug. The following section will therefore summarise the employed methods using the same differentiation.

Methods for the Characterisation of the Lipids and Colloidal Phases

Quantification of the different digestion products, including mono-, di-, triglycerides, fatty acids, bile salts and phospholipids can be carried out by use of HPLC, GC or HPTLC (69,86). This provides an alternative to the indirect measure of digestion by NaOH consumption (69,86).

Table III. Recent Techniques Employed for the Characterisation of *In Vitro* Lipolysis

In vitro lipolysis	Substrate/drug	Technique	Comment	References
Phosphate buffer at pH 6.8, 10 mM bile salts from bile extract 10 mM Ca^{2+} , porcine pancreatin extract and 150 U/ml lipase activity	1.5% olive oil emulsion (15 mM triolein)/1 mM tempol benzoate	Drug—EPR spectroscopy, lipids and digestion; products—HPTLC	Quantification of spin label in digestion phases; quantification of lipids by HPTLC	(87)
Tris buffer at pH 7.4, 13 mM NaTDC, 8 mM Ca^{2+}, porcine pancreatin extract and 20 U/mg lipase activity	5% oil emulsion (glyceryl trioctanoate and glyceryl trioleate)/ergosterol, progesterone and vitamin D_3	Multiplex CARS microspectroscopy	Label-free imaging and quantification of drug and lipid digestion products	(88)
Tris maleate at pH 6.5, 5 mM bile salts from porcine bile extract, continuous Ca^{2+} addition (0.045 mM/min), porcine pancreatin extract, 350 U/ml lipase activity	1 g SNEDDS—55% medium-chain and long-chain lipids, 35% CrRH40 and 10% ethanol	Cryo SEM	Structure of dispersed SNEDDS	(37)
tris maleate pH 6.5, 5 mM bile salts from bile extract, continuous Ca^{2+} addition (0.045 mM/min), porcine pancreatin extract, 800 U/ml lipase activity	3 g SNEDDS—30% sesame oil, 30% mixed LC glycerides, 30% CrRH40 and 10% ethanol	Cryo TEM	Structures of dispersed SNEDDS and digestion products	(56)
Tris maleate pH 7.5, 5 mM/20 mM NaTDC, 5 mM Ca^{2+}, porcine pancreatin (8× USP) and 50 TBU/ml	0.25 g medium-chain or long-chain lipid	HPTLC	Quantification of mono-, di- and triglycerides and fatty acids during digestion	(86)
Tris maleate at pH 6.5, 5 mM bile salts from bile extract, continuous Ca^{2+} addition (0.045 mM/min), porcine pancreatin extract and 800 U/ml lipase activity	3 g SNEDDS—30% sesame oil, 30% mixed LC glycerides, 30% CrRH40, and 10% ethanol	Bench-top SAXS and cryo TEM	Structures of dispersed SNEDDS and digestion products	(82)
Tris maleate pH 7.5, 5 mM NaTDC, 10 mM Ca^{2+}, porcine pancreatin (8× USP), 20,000 TBU	Phytantriol, glyceryl monooleate, SNEDDS—30% sesame oil, 30% mixed LC glycerides, 30% CrRH40 and 10% ethanol	Synchroton SAXS	Real-time observation of dispersed SNEDDS and digestion products	(89)

Cryo SEM/TEM cryogenic scanning/transmission electron microscopy, *EPR* electron paramagnetic resonance, *HPTLC* high-performance thin layer chromatography, *NaTDC* sodium taurodeoxycholate, *SAXS* small angle X ray scattering, *SNEDDS* self-nanoemulsifying drug delivery system, *TBU* Tributyrin unit (1 TBU equals the amount of enzyme that can liberate 1 mol of fatty acid from tributyrin/min (69))

The bulk structure of diluted SNEDDS has commonly been investigated by dynamic light scattering (DLS) (56,90). In DLS an incident laser beam is scattered by dispersed particles in a sample. The fluctuation in the scattering signal, caused by Brownian motion of the particles, is measured as a function of time. From the correlation function, the particle size is calculated as an approximation from the diffusion coefficient. Although DLS does not allow a direct size measurement, it is a convenient and fast screening tool for particle size measurements including SNEDDS. However, other methods such as electron microscopy and X-ray scattering techniques (discussed below) might provide complementary information.

The rapid immobilisation of aqueous dispersions of SNEDDS by plunge-freezing in liquid nitrogen allowed the preservation of the dispersed particle in the bulk. The subsequent cryogenic electron micrographs of the frozen samples revealed the spherical shape of the dispersed SNEDDS and particle sizes between 30 and 40 nm (37).

Similarly, the utilisation of cryogenic transmission electron microscopy (cryo-TEM) with its superior resolution compared with cryo-SEM allowed the observation of morphological changes of SNEDDS during *in vitro* lipolysis (56,82). Before lipolysis was initiated, the medium was dominated by the presence of oil droplets (approximately 50 nm) and micelles formed by bile salts and phospholipids (approximately 5–10 nm). With progressing lipid digestion, unilamellar and multilamellar colloidal structures emerged at the expense of oil droplets while micelles were constantly observed. However, after 30 min *in vitro* lipolysis the number of the unilamellar vesicles declined and multilamellar vesicles disappeared. This behaviour was explained by the likely incorporation of the generated digestion products in micelles, thus forming swollen mixed micelles.

In order to elucidate the structures of the phases observed with cryo-TEM, small-angle X-ray scattering (SAXS) has been used during *in vitro* lipolysis of SNEDDS. SAXS is a method taking advantage of the scattering of X-rays impinging on macromolecules or self-assembled structures in the nanometre to micrometre range using scattering angles smaller than 10° (2Θ) for the detection of the scattered X-rays (91). This method revealed the steady development of a lamellar phase followed by the co-existence of lamellar and inverse hexagonal phases, the latter prevailing at the end of *in vitro* lipolysis (Fig. 3).

It should be noted that for the described cryo-TEM and SAXS experiments, samples were withdrawn during lipolysis, providing snapshots of the dynamic process. Recently, online monitoring of the lyotropic phases generated during lipolysis of the same SNEDDS as described above was reported (92). Using synchrotron radiation the phases identified with bench-top SAXS were confirmed. Differences were observed in the temporal appearance of the phases which could be traced back to the slightly different lipolysis protocols: with the bolus addition of calcium a hexagonal phase was detected by synchrotron SAXS within 5–10 min whereas the same phase was observed from 60 min using continuous calcium addition and bench-top SAXS (Fig. 3).

Methods for the Characterisation of the Drug

With regard to the drug, the analytics carried out during *in vitro* lipolysis comprises the quantification by HPLC in the supernatant and pellet obtained after a centrifugation step (63,65). Traditionall,y samples of the lipolysis medium have been subjected to ultracentrifugation. In a recent systematic study, the separation of digestion phases of less lipophilic formulation by ultracentrifugation was comparable to conventional bench-top centrifugation enabling a higher sample

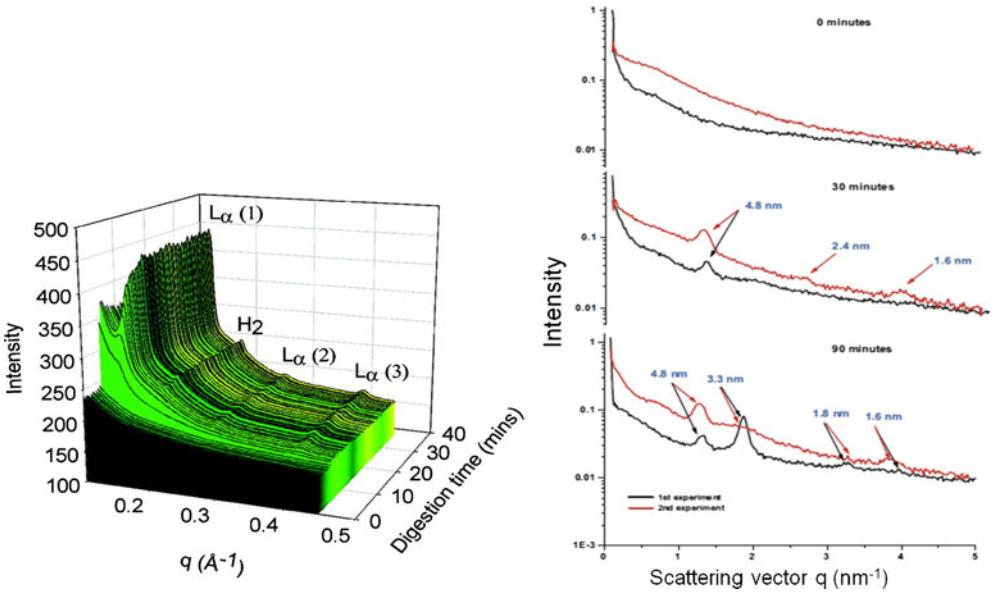

Fig. 3. SAXS data using either synchrotron (*left*) or bench-top SAXS (*right*). The intensity of the scattered electrons is plotted *versus* the scattering vector (q), for synchrotron also *versus* time. The spectra illustrate the evolution of lamellar (L_α) and hexagonal (H_2) phases during *in vitro* lipolysis. The arrows on the right panel denote the Bragg spacing for the lamellar phases at day=1.6, 2.4, and 4.8 nm and at day=3.3 nm for the hexagonal phase. At time points 0 min, no liquid crystals are visible, after 30 min a lamellar phase exists, and 90 min hexagonal and lamellar phases co-exist. Reprinted with permission from references (82,89)

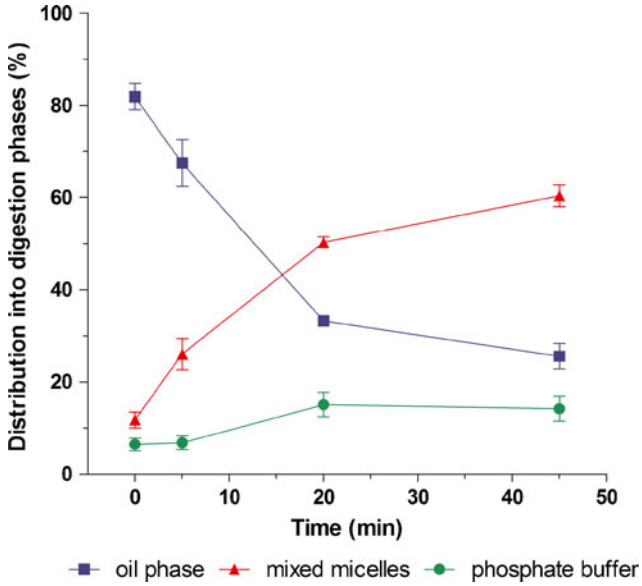

Fig. 4. Distribution of the spin probe tempol benzoate during *in vitro* lipolysis of 1.5% (*v/v*) olive oil as determined by electron paramagnetic spin resonance spectroscopy, EPR, in the presence of 10 mM bile acids. Redrawn and adapted from Rübe *et al.* (87)

turn-over for laboratories (93). However, the presence of an oil layer in case of more lipophilic LBDDS required ultracentrifugation for the effective separation of oil, aqueous phase, and pellet.

It is assumed that the drug solubilised in the aqueous phase is the fraction available for absorption, whereas precipitated drug has been considered undesirable since the solid drug requires a dissolution step potentially reducing bioavailability (2,57).

Recently, the solid state properties of the precipitates generated during *in vitro* lipolysis have gained increased interest. When the pellet obtained after ultracentrifugation of a SMEDDS subjected to *in vitro* lipolysis was investigated using XRPD and polarising light microscopy, the results indicated that the model drug cinnarizine precipitated in an amorphous form showing a much faster dissolution rate compared with the crystalline drug (94). In a similar study, amorphous halofantrine precipitated during *in vitro* lipolysis of two supersaturated SNEDDS based on either medium- or long-chain lipids (95). Despite substantial precipitation during *in vitro* lipolysis the bioavailability of halofantrine was not negatively affected after the administration of the same formulations to beagle dogs. Amorphous drug precipitation was recently shown for ketoconazole in intestinal aspirates (96). Assuming halofantrine precipitated also in an amorphous form *in vivo*, the rapid dissolution rate of the amorphous drug (as seen *in vitro*) could explain the retained bioavailability. From these initial studies, it appears that the propensity of drug to precipitate *in vitro* could be a misleading parameter for *in vivo* predictions of LBDDS without considering the solid state properties of the precipitate. Therefore, well-established solid state characterisation techniques might find increasing application for the assessment of lipid-based formulations providing valuable information supplementing *in vitro* lipolysis.

During *in vitro* digestion the co-administered drug partitions between the continuously changing colloidal phases which have different solubilisation capacity for the drug (84,97). The current standard method employed for the separation of the digestion phases are conventional centrifugation and ultracentrifugation which provide only an unrefined picture of drug distribution. The spatio-temporal patterns of drug distribution in the lipolysis medium might be more accessible by complimentary methods.

One of these methods is electron paramagnetic resonance (EPR) spectroscopy, also known as electron spin resonance spectroscopy. Similar to nuclear magnetic resonance, a magnetic moment ('spin') is mandatory for EPR spectroscopy. In the case of EPR, unpaired electrons are excited by a micro- or radio-wave source in a changing magnetic field (98). When in resonance the electron spin reverses, causing some of the electromagnetic radiation to be reflected which corresponds to the EPR signal. The signal is sensitive to alterations in the microenvironment (such as in the polarity) of the paramagnetic compound. As the polarity in the direct vicinity of a compound changes during *in vitro* lipolysis, EPR spectroscopy was successfully employed to monitor the distribution of the lipophilic, paramagnetic compound tempol benzoate (TB) between undigested oil, aqueous phase and mixed micelles (87). The dynamics of drug distribution was most evident during the initial 20 min of *in vitro* lipolysis (Fig. 4), corresponding to the digestion of approximately 30% of the oil (as determined by HPTLC). Although a promising, non-invasive technique, a more widespread utilisation of EPR might be restricted due to the requirement of a paramagnetic compound or the need for adequate labelling.

In contrast, coherent anti-Stokes Raman scattering (CARS) microspectroscopy does not rely on a specific label (99). Images are obtained from molecular Raman vibrations which render the method highly chemical-specific. CARS spectroscopy is a coherent method resulting in signal strengths several orders of magnitude greater than spontaneous Raman scattering (100). The advantage of high sensitivity and spatial resolution has sparked the interest in CARS as a non-invasive tool to probe biological samples such as living cells in real time (99). Recently, multiplex CARS microspectroscopy has been employed to characterise *in vitro* lipolysis (88). The method was

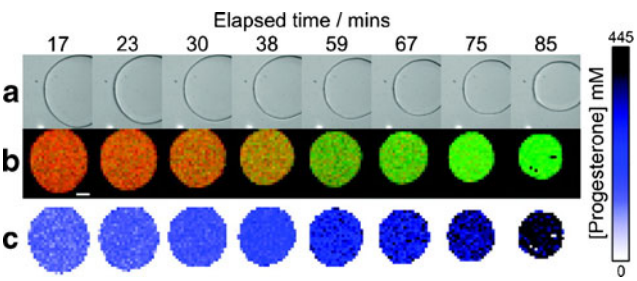

Fig. 5. The progress of triglyceryl trioctanoate digestion by pancreatic lipase observed over time by bright field microscopy (**a**) and CARS (**b**,**c**). False colour images of triglyceryl trioctanoate (*red*) and progesterone (*green*) revealed diminishing triglyceride content and increasing progesterone concentration. The concentration of progesterone (*blue*) is provided in the colour bar, corresponding to the false colour images of progesterone within the lipid droplet (**c**). The initial progesterone concentration was 95 mM, the scale bar equals 10 μm. Reprinted with permission from Day *et al. (93)*. Copyright (2010) American Chemical Society

suitable to locate and quantify several lipophilic drugs during progressing lipolysis. As an example, from the increasing concentration of progesterone in the diminishing oil droplets (Fig. 5c), the authors concluded that the investigated vehicle was ill-suited as a delivery system for progesterone since the drug did not distribute into the digestion products, probably due better solubility in the triglyceride.

An additional benefit of CARS arises from its ability to visualise and quantify the digestion of the lipids (88) (Fig. 5b). Further improvements might enable this promising technique to be conducted also under more realistic hydrodynamic conditions and *in vivo*. This would eventually provide insights into the mechanisms of drug and lipid absorption on a cellular level.

CONCLUSIONS

Lipid-based drug delivery system has become increasingly popular for the delivery of poorly water-soluble, lipophilic compounds due their potential to improve the bioavailability of these drugs. So far, a rational design for these delivery systems has not yet been established. The current work tried to summarise the established methods and show the trends and pitfalls in the characterisation of *in vitro* lipolysis indicating the need for a multi-disciplinary approach which seems to be the key to further our knowledge about these promising delivery systems.

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