Advanced Drug Delivery Reviews 142 (2019) 50–61

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Drug supersaturation during formulation digestion, including real-time analytical approaches

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ARTICLE INFO

Article history:
Received 22 August 2018
Received in revised form 6 November 2018
Accepted 9 November 2018
Available online 13 November 2018

Keywords:
Self-emulsifying
Lipid-based
Lipolysis
Drug supersaturation
Precipitation
Real-time

ABSTRACT

Self-emulsifying and other lipid-based drug delivery systems have drawn considerable interest from pharmaceutical scientists for managing oral delivery of poorly water-soluble compounds. Following administration, self-emulsifying systems exhibit complex aqueous dispersion and digestion in the gastro-intestinal tract. These processes generally result in drug supersaturation, which leads to enhanced absorption or the high drug concentrations may cause precipitation with erratic and variable oral bioavailability. This review briefly outlines drug supersaturation obtained from self-emulsifying and other lipid-based formulations; recent advancements of *in vitro* lipolysis testing are also discussed. Further, a main focus is mechanisms by which supersaturation is triggered from gastro-intestinal processes, as well as analytical techniques that are promising from a research and development perspective. Comparatively simple approaches are presented together with more sophisticated process analytics to enable direct examination of kinetic changes. The analytical methods together with their sensor probes are discussed in detail to clarify opportunities as well as technical limitations. Some of the more sophisticated methods, including those based on synchrotron radiation, are primarily research oriented despite interesting experimental findings from an industrial viewpoint. The availability of kinetic data further opens the door to mathematical modeling of supersaturation and precipitation *versus* permeation, which lays the groundwork for better *in vitro* to *in vivo* correlations as well as for physiologically-based modeling of lipid-based systems.

Contents

1. Introduction

Lipid-based formulations (LBF) have been used in the pharmaceutical sciences for approximately half a century [1] and are of growing importance, attributable to a rising number of poorly soluble drug candidates [2]. Unfavorable drug solubilization properties, such as large molecular size and high lipophilicity, are often the outcome of high-throughput screening, as well as the use of chemical libraries; further, they are dependent on the physico-chemical nature of the drug targets [3]. Pharmacophores generally have molecular requirements of size and lipophilicity that come together with many other limitations to obtain safe and efficacious drug candidates. Therefore, medicinal chemists can only optimize molecules within given constraints.

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https://doi.org/10.1016/j.addr.2018.11.003
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Further, development candidates are often beyond class I of the biopharmaceutics classification system (BCS) [4], meaning they are either poorly water-soluble and/or show poor intestinal permeability. Unfavorable biopharmaceutical drug properties emphasize the importance of selecting a viable oral formulation strategy such as development of self-emulsifying systems [5].

Poorly soluble drugs that are based on a solvation limitation [6] are typical candidates for lipid formulations. Hydrophobic compounds have high crystal lattice energy and, therefore, exhibit poor solubility in aqueous media as well as in other solvents and lipids [7]. Currently, LBFs are here not the first formulation of choice but recent advances in ionic liquid technology may change this in the future [8–9]. However, the suitability of LBFs using ionic liquid technology is dependent on the availability of excipients that are pharmaceutically acceptable from a safety and regulatory perspective.

In contrast to LBFs that use ionic liquid technology, there are a wider range of established excipients for more standard LBFs that have a compendial status, enabling formulations of different polarity and dispersion characteristics. Formulations have been assigned to different categories in the lipid formulation classification system (LFCS), which was initially coined in 2000 [10] and later updated in 2006 [11]. Systems can be rather simple, such as oils (class I), or mixtures formulated with surfactants and co-solvents leading to self-emulsifying drug delivery systems (SEDDS). It is possible to obtain nano-sized droplets that are mostly nano-emulsions (SNEDDS as concentrates), whereas true microemulsions (SMEDDS as concentrates) are rarely obtained [12]. Differences between SNEDDS and SMEDDS are of academic interest for the formulation nomenclature; however, they are not expected to have biopharmaceutical relevance.

The biopharmaceutical performance of LBFs is often greatly enhanced over that of simple crystalline formulations of poorly water-soluble drugs [13]. As such, LBFs comprise drugs in a dissolved form, which circumvents a dissolution step and is likely a dominant reason for the improved performance. However, it is important that such formulations maintain the drug in a solubilized form to facilitate absorption. Alternatively, a drug may crash out during dilution and dispersion phase changes or as triggered by changes in digestion. Therefore, a good understanding of gastro-intestinal formulation processing is important for adequate development of LBFs [14]. It is especially critical to understand the effects of solubilization and supersaturation, as the latter is the driving force for drug precipitation [15]. Thus, excessive supersaturation should be avoided, and high intestinal drug concentrations may be reached for LBFs using a mixture of moderate supersaturation in combination with enhanced solubility.

Based on their ability to generate drug concentrations beyond the solubility limit, LBFs are viewed as effective supersaturable dosage forms, which is similar for other systems like, for example, solid dispersions [16]. Fig. 1 depicts different mechanisms taken from the literature that are known to enhance oral drug absorption and bioavailability [17–19]. The upper three mechanisms, including increased solubilization, generation of supersaturation, and inhibition of re-crystallization, are the focus of this review together with "real-time" techniques that can measure kinetic changes *in vitro*. The other listed mechanisms are primarily biological effects of lipid-based excipients or of their formulations and are not within the scope of this review; however, they may still be relevant for many drugs. Therefore, the listed mechanisms (Fig. 1) provide a reminder that LBFs should not only be viewed as a supersaturable dosage form, as lipid formulations have a more complex spectrum of possible influences in the gastro-intestinal tract.

2. Apparent and true drug supersaturation

The kinetics of gastro-intestinal drug concentrations are schematically depicted in Fig. 2. High concentrations can be obtained for LBFs, whereas crystalline drugs typically have slow dissolution kinetics when approaching the solubility limit [1,20]. The high drug solubilization of LBFs is a supersaturation effect; however, there is also a further increase in the solubility limit in the presence of lipids. Fast dispersion occurs in parallel to rapid initial drug release for self-emulsifying systems. Released compound is here mostly understood as solubilized drug in an aqueous colloidal phase that essentially results in an apparent concentration. In contrast, a stricter view considers only free drug as truly being released. This initial increase in concentration beyond the thermodynamic solubility limit has been termed "spring", whereas a "parachute" is the ability to sustain drug supersaturation as outlined in Fig. 2 [21]. The latter concept of supersaturation is often understood as a supersaturation ratio S, as given in Eq. (1):

$$S = \frac{C}{C^*} \tag{1}$$

where c is the (molar or mass) concentration of supersaturated drug and c^* denotes the equilibrium solubility. It is practical to use concentrations instead of the more correct thermodynamic activities, as the latter

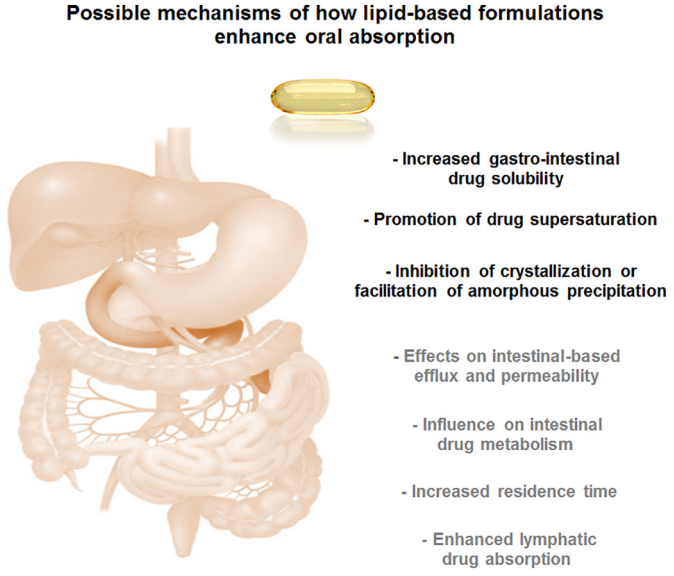

Fig. 1. Different mechanisms are listed that can increase oral drug absorption from lipid-based drug delivery systems [17].

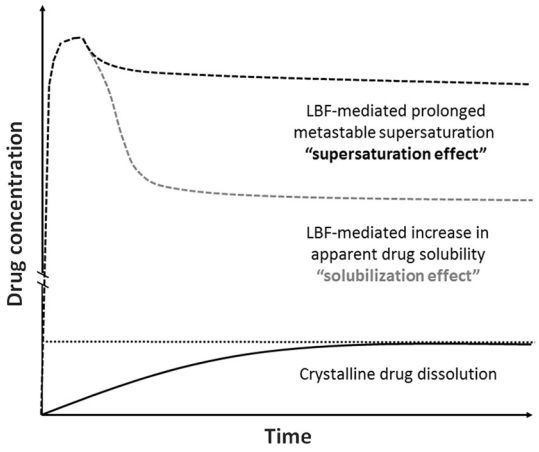

Fig. 2. Idealized concentration profiles of how lipid-based formulations (LBFs) can solubilize a poorly water-soluble drug in the gastro-intestinal tract. There is an effect of the excipients on drug solubility, which is depicted by the dashed gray line. This typically comes with an additional "supersaturation effect", as excipients and endogenous colloids can sustain metastable concentrations. The thermodynamic solubility of such a compound is lower, and this equilibrium would be approached only slowly by dissolution of the crystalline form (Figure adapted [1,20]).

are not as easily accessible. Activity a is linked to mole fraction concentration x *via* an activity coefficient γ, as shown in Eq. (2):

$$a = \gamma x \tag{2}$$

For example, when the risk of precipitation from a supersaturated solution is considered, supersaturation is a direct measure of the chemical potential difference in solution and in the crystal phase, $\Delta\mu$ [22]:

$$\Delta\mu = RTln\left(\frac{a}{a^*}\right) \tag{3}$$

where R is the universal gas constant, T is the temperature, and the bracketed supersaturation ratio is drug activity a (in the supersaturated solution) divided by the activity at equilibrium a^*. This equation demonstrates the driving force of precipitation proportional to the logarithm of supersaturation (*i.e.*, supersaturation ratio) based on activities. It depends on the given activity coefficients how accurate supersaturation can be expressed based on mass or molar concentrations. Supersaturation drives drug precipitation and further enhances diffusion and permeation across the intestinal wall. At high concentrations in water, liquid-liquid phase separation (LLPS) eventually occurs, as observed when drugs "oil out", resulting in the free compound dominating the permeation flux or precipitation [23]. Here, solute activity may clearly be different from the (nominal) concentration c, which can be studied *via* experiments of drug flux through a membrane [24]. Such experiments particularly demonstrate how the presence of solubilizing excipients affect activity as well as absorptive flux [25]. While LLPS leads to a plateau in maximum activity, surfactants are a further limiting factor for drug activity, mostly attributable to partitioning with the aqueous bulk phase. Fig. 3 depicts a model drug (cinnarizine) on the surface of a vesicle as a molecular snapshot of partitioning between the colloid and bulk phase. Such molecular interactions lead to different populations of drug inside or on the surface of colloids, as well as a free bulk fraction. The latter population of molecules is primarily relevant for membrane permeation; it is helpful to think of colloidal solutions as heterogeneous media in which a drug can find different molecular environments. For drug precipitation, this means that nucleation sites likely occur wherever high local activities are given for sufficiently long times. Therefore, for comparatively lipophilic compounds, a preferential site for drug precipitation is inside colloidal or oily droplets, whereas other hydrophobic drugs may crystallize on surfaces or even in bulk. The distribution coefficient of a drug may provide information regarding where precipitation will likely occur, while apparent supersaturation is a viable indicator of drug precipitation [23].

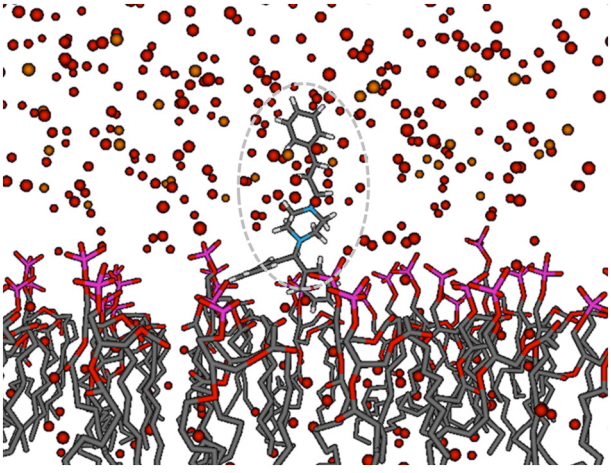

Fig. 3. Schematic representation of a model drug (*i.e.*, cinnarizine) that interacts with a colloidal surface. Ions are shown, and water is depicted without hydrogens for clarity of presentation. The total drug concentration in such heterogeneous systems should be differentiated from the thermodynamic drug activity. The graph was based on a molecular simulation snapshot using the software program ChemSite Pro. v. 10.4 (Norgwyn Montgomery Software Inc., North Wales, USA).

The mentioned colloidal species are formed by excipients of the formulation; however, they also emerge from endogenous amphiphiles such as phospholipids and bile salts. An artificial version of this colloidal solution is the well-known fasted-state simulated intestinal fluid, FaSSIF [26]. Effects of drug partitioning in such endogenous micelles have been discussed by Sugano et al. [27]. Mechanisms underlying the molecular features of drug influences, such as micellar affinity and solubilization, are of interest. The partitioning coefficient, $\log(K_m)$, is obtained from the logarithmic solubility enhancement of drug in colloidal medium and the solubility in medium without micelles ($\log(SE)$). Partitioning from the pseudo-phase of the micelles and the bulk phase can be modeled using a linear free energy relationship [28]:

$$\log(K_m) = \log(SE) = c + eE + sS + aA + bB + vV \tag{4}$$

where coefficients are included together with different molecular properties that are denoted using a capital letter. Thus, A is hydrogen-bonding acidity and B (or also referred to as B_0 in condensed phases) is basicity, while S describes dipolarity/polarizability. Moreover, E is the excess molar refraction descriptor, and V represents McGowan characteristic volume. A detailed description of these molecular properties is given in the literature [29–30], and the approach used for Eq. (4) has been recently applied to model drug solubilization in FaSSIF [31]. This biorelevant solubility enhancement compared to buffer ($\log(SE)$) revealed especially pronounced effects of two molecular properties (Fig. 4). One was a positive component effect of McGowan volume (V) and another strong influence on $\log(SE)$ was from drug basicity (B_0). The former volume effect is readily understood when imagining a molecular cavity in the solvent environment, which is required to solvate a rather lipophilic drug. On the other hand B_0 can be viewed regarding hydrogen bonding of basic groups in aqueous medium, which is suppressed in a lipophilic environment such as a micellar core. The linear free energy relationship yielded a correlation coefficient of 0.90 for the tested 40 poorly soluble drugs [31] and it seems that good *in silico* prediction is just one benefit of this modeling approach, while another is to gain an improved molecular understanding of which properties are driving biorelevant solubilization.

According to Eq. (4), drug partitioning and, hence, the free fraction greatly depend on the individual physico-chemical properties of the drug and surrounding medium. The presence of excipients in the medium is expected to affect coefficients; interestingly, the research group of Anderson [32] demonstrated how water uptake in triglyceride/monoglyceride microemulsions influenced the coefficients of such a linear free energy relationship. The obtained coefficients showed a systematic dependence on lipid composition and water uptake. This supported the view that relative solubility was determined largely by molar concentrations of individual functional groups such as glyceride esters moieties and hydroxyl groups. When considering lipolysis of LBFs, there would be continuous changes in the medium composition that are more complex than a simple dilution of the relevant functional groups. Accordingly, this greatly complicates the situation for any mathematical modeling of drug solubilization.

Given the complexity of dynamic medium changes, it can be asked how problematic it is to use apparent supersaturation instead of a supersaturation based on activities? The answer lies in the usefulness of the given approach to describe data and to make sense of it. As discussed previously, apparent drug supersaturation can be a suitable marker for drug precipitation. Interestingly, supersaturation has only recently been applied to research of lipid-based drug formulations, which has resulted in a major step forward from previous approaches focusing only on drug solubility as obtained from LBF dispersion and digestion.

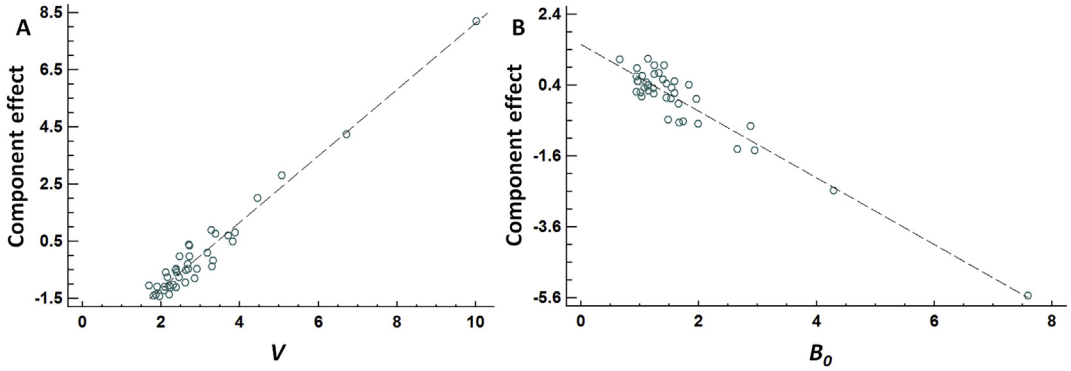

Fig. 4. The solubility enhancement in FaSSIF compared to buffer (log(*SE*)) is modeled by a linear free energy relationship (in line with Eq. (4)) and the most important component effects are shown, *i.e.* McGowan volume *V* and molecular basicity B_0.

3. Use of the apparent supersaturation ratio in LBF dispersion and digestion

3.1. Solubilization and supersaturation aspects of lipid-based systems

During the early years of lipid-based drug delivery research, supersaturation was viewed already as the cause of drug precipitation; however, it was not typically directly studied, and studies focused on the emerging precipitation and solubility *in vitro* [33–34]. Later, researchers at Monash University specifically considered *in vitro* lipolysis-triggered drug supersaturation and how it affected *in vivo* performance [35]. This pioneer study introduced a maximum degree of supersaturation, S^M, for dispersion and digestion. The ratio of the potential maximum drug concentration in aqueous colloidal phase (AP, in the absence of drug precipitation) and the equilibrium solubility in the AP (obtained after dispersion/digestion of a drug-free formulation) was taken as a measure of precipitation risk. While the initial article by Anby et al. [35] roughly estimated kinetic changes by determining S^M after 5 and 60 min of digestion, other work typically used a single reference time (*e.g.*, 30 min) to estimate the maximum supersaturation ratio [36]. Empirically, it was observed that the risk of *in vitro* precipitation increased sharply once S^M values were greater than about three. Some formulations were found to be supersaturated during dispersion; however, depending on the individual system, the process of digestion was found to greatly contribute to S^M. Lipid type was associated with interesting effects; for example, long chain lipids did not develop pronounced supersaturation, which differed from LBF-containing medium chain lipids for which most precipitation was observed, as in the case of danazol, a model drug. The effects associated with lipid type were not specific for danazol; however, this phenomenon appeared to apply to various poorly soluble drugs [37]. Medium chain lipids typically solubilize comparatively more drug in the formulation than long chain lipids; however, once dispersed and digested, a high colloidal solubilizing capacity is achieved with long chain lipids. Evidently, solvation effects attributable to chain length in the undispersed and dispersed state define final drug supersaturation. A similar study concluded that the extent of supersaturation on dispersion is dependent on the hydrophilicity of the formulation components, whereas the extent of supersaturation on digestion mirrored digestibility of the LBF as well as the solubilization capacity of the digestion products [38]. This general assumption was further supported by research at Monash University comparing different LBF surfactants [39]. Moreover, further work proved the utility of a maximum theoretical supersaturation to understand surfactant effects on drug precipitation *in vitro* [40].

Considerations of LBF supersaturation have advanced the field, and early work suggested the practicality of this approach for explaining *in vivo* data [35]. A recent study of LBFs using a cholesteryl ester transfer protein inhibitor found a suitable correlation with *in vivo* data when apparent supersaturation at 30 min *in vitro* digestion (S_R) was considered [41]. Two approaches for correlating the *in vitro* digestion data to exposure in dog studies are depicted in Fig. 5. A single, linear correlation was obtained with different formulations of the surfactant Kolliphor RH 40 [41]; however, the correlation did not extend to formulations based on other surfactants. Therefore, a consideration of solubility in AP and S_R alone was apparently not sufficient to explain drug exposure across very dissimilar formulations.

Studies using the drug fenofibrate reported that *in vitro* digestion failed to predict the *in vivo* rank order of formulations [42–43]. Clues for explaining this lack of correlation were obtained from two important studies. First, an *in vivo* precipitation study conducted in rats at the University of Copenhagen did not detect crystalline fenofibrate [44] in the intestine. Another study at Monash University [45] introduced an *in vitro* digestion- *in vivo* absorption model and also concluded that a simple *in vitro* digestion test may over-predict the extent of fenofibrate precipitation *in vivo*. The dynamic situation *in vivo* leads to a parallel digestion and absorption of released drug so that intestinal supersaturation is theoretically expected to be lower than that observed from an *in vitro* lipolysis test without a sink [46].

Apart from the aspect of an absorption sink, a further important factor is the solid state of the precipitate. Following *in vitro* digestion, it is interesting to study whether crystalline drug is present in the formed pellets; for example, X-ray diffraction experiments could be conducted. A study using cinnarizine LBFs suggested that crystalline base was not found in the pellet [47]. A lack of crystalline drug is also evidenced by studies using the base halofantrine, for which so-called "super-SNEDDS" were formulated [48]. In these studies, halofantrine was already supersaturated in the formulation itself, and the increased loading yielded a comparatively more pronounced supersaturation following dispersion and digestion. The amorphous state of the precipitate from super-

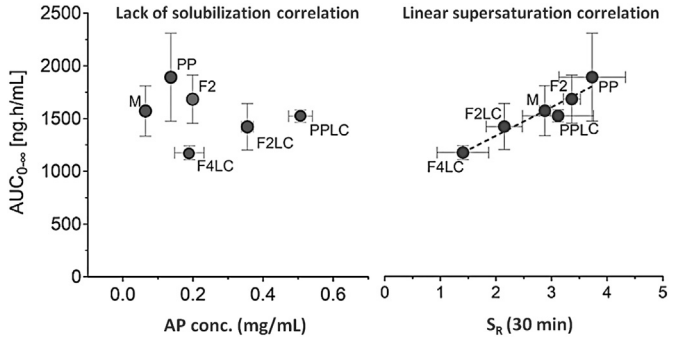

Fig. 5. *In vitro* data of a cholesteryl ester transfer protein inhibitor were correlated with the area under the plasma curve (AUC) in beagle dogs after administering different lipid-based systems. Drug solubilization in the aqueous phase (AP conc.) for different formulations (containing Kolliphor RH 40) did not reveal a pronounced correlation; however, a nice linear relationship was found for drug supersaturation after 30 min. Digestion *in vitro* (details are given in the text; Figure adapted [41]).

SNEDDS resulted in fast re-dissolution (*in vitro*) and this finding together with a pharmacokinetic study in beagle dogs supported the view that absorption of halofantrine was not hampered by drug precipitation.

Amorphous drug precipitation was further explored by comparing two model bases, carvedilol and loratadine, both of which have similar lipophilicity [49]. X-ray diffraction studies of the precipitates indicated that carvedilol precipitates in a crystalline form upon dispersion; interestingly, this drug resulted in an amorphous precipitate during lipolysis. In contrast, loratadine precipitated as a crystalline material during both formulation dispersion and digestion. Studies have suggested that carvedilol forms strong molecular interactions with oleic acid [50]; therefore, researchers have hypothesized that fatty acids produced during lipolysis could affect the solid form of the precipitate. This idea sparked a thorough solid-state investigation of isolated pellets from *in vitro* digestion of cinnarizine formulations [51]. Results based on differential scanning calorimetry (DSC) as well as solid state ^{13}C and liquid state ^{1}H nuclear magnetic resonance spectroscopy (NMR) complemented each other and supported the assumption of molecular interactions between fatty acids and drugs, which in turn would favor precipitation of non-crystalline cinnarizine. A lipolysis study with a focus on the solid state of precipitated cinnarizine suggested that such a drug base can interact with fatty acids to form amorphous precipitates (below pH 8.0) [52]. More research is certainly needed with further compounds and formulations to better understand in which cases amorphous precipitates are obtained during digestion because of fatty acid interactions with drug bases. Based on the recent findings, studies on the solid state of any precipitation that occurs *in vitro* are encouraged [46,53].

3.2. Advancements of in vitro lipolysis testing

Analyses of the given solid form in which a drug precipitates help extract more information from *in vitro* lipolysis testing. Further advancement of classical lipolysis testing can go in different directions. A first direction is about experimental improvements to make *in vitro* lipolysis more physiologically relevant, which inevitably comes with a higher level of sophistication. By contrast, another trend of current research is rather about simplification and parallel testing. Finally, a third direction involves advancements in analytical development and modeling to better study the dynamics of lipolysis as well as drug supersaturation. The first two research directions are discussed here only briefly, whereas this article discusses the analytical developments in more detail in subsequent sections.

In vitro lipolysis testing based on pioneer research in Copenhagen and at Monash University was already used in pharmaceutics shortly after the turn of the millennium [54–55]. This classical test served to study effects of lipolysis on formulation changes; however, there are limits to predicting the relevance *in vivo* when pertinent mechanisms of absorption are not accounted for, which was previously discussed. An important aspect is the initial passage through the acidic stomach where gastric digestion takes place [56]. Early work by Jannin et al. and Carrière et al. [57–58] tried to better simulate digestion of formulations *in vivo* using gastric lipolysis, which was later utilized by the lipid formulation classification system (LFCS) consortium [59]. The release of weak bases as protonated drugs in solution can generate excessive supersaturation once the higher pH of the intestine is reached, making inclusion of a simulated gastric step particularly interesting to study. A study using cinnarizine with a gastric step during lipolysis testing compared a conventional tablet, a SNEDDS, and a solidified SNEDDS [60]; the aqueous phase of the simulated stomach displayed not only for the SNEDDS high concentrations but also for the tablet due to fast release at the low pH. The main difference was then the parachute effect of the LBF in the intestinal medium, which was missing for the tablet. The solidified SNEDDS displayed concentration in the aqueous phase that were between those of the other formulations for both, the simulated gastric and intestinal step [60]. This observed formulation ranking in the fasted state agreed well with results from oral bioavailability studies in dogs.

Apart from a missing gastric lipolysis step, the classical lipolysis model has the disadvantage of a lacking absorption sink. This is especially a critical factor for poorly soluble but highly permeable drugs such as fenofibrate, which do not generate such high supersaturation states *in vivo*, as observed during non-sink lipolysis testing. It was previously mentioned that the introduction of an *in vitro* digestion-*in vivo* absorption model marked a substantial advancement from a research perspective [45]. A benefit of these rat jejunal perfusion experiments is that a realistic permeation flux of drug can be observed, and this approach has recently been applied to studies on the effects of polymeric precipitation inhibitors on fenofibrate supersaturation from hydrophilic LBFs [61].

Compared to *in vivo* perfusion experiments, other *in vitro* approaches typically struggle with a realistic sink; however, they have the advantage of avoiding elaborate *in vivo* experiments, including animal surgery. Interestingly, development of lipolysis tests at the University of Uppsala combined a cell-based permeation assay with *in vitro* lipolysis [62]. Most of the digested formulation components were quite compatible with the employed Caco-2 cells; however, pancreatic enzyme was not tolerated by the cells. Immobilized pancreatic lipase can be, however, used and mucin had beneficial effects on the tolerability of more critical formulation components regarding cell viability.

Cellular sensitivity can be circumvented by using a membrane, and Permeapad®, a biomimetic barrier, was tested for this purpose in a combined digestion-permeation model [63]. The marker calcein was first studied to confirm that the barrier maintained its integrity during lipolysis. Subsequent studies using the model drug cinnarizine formulated as SNEDDS were promising; however, more research is needed to determine correlations with *in vivo* formulation performance. The potential to run parallel tests in the future using a biomimetic membrane is very attractive from an industrial viewpoint.

The need of the pharmaceutical industry to obtain high-throughput testing was leading to a separate direction to advance lipolysis testing for which simplicity and miniaturization are in the focus. The apparent S^M in the aqueous phase of *in vitro* lipolysis tests can be a useful marker of drug precipitation risk from formulation despite theoretical limitations that may often lead to failures in predicting *in vivo* exposure, as previously discussed. One may argue that given all this incertitude, S^M should be obtained from a simpler test at an early stage of pharmaceutical development. One idea is to use a high buffer capacity instead of a pH-stat titration system, which enables a simplified *in vitro* digestion test with the potential for high-throughput experimentation [64–65]; this is similar to the reliance on solubility testing in simulated media when estimating S^M. This approach is certainly a simplification, as it does not account for any dynamics during lipolysis. However, simplicity and good expected reproducibility are the likely advantages of this approach.

It is easy to show from the definition of apparent supersaturation that drug solubility in the digested aqueous phase (or artificially digested medium), c^*_{digest}, together with solubility in the formulation, c^*_{lipid}, provide a ratio that is proportional to the apparent S^M [37]:

$$S^M = \frac{S_{lipid}}{\kappa} \frac{c^*_{lipid}}{c^*_{digest}} \tag{5}$$

The proportionality to S^M holds true if formulations are compared with the same saturation level (S_{lipid}) and constant volume factor of medium dilution κ. Results of various compounds that were studied using this approach based on simple caprylic triglycerides at an initial saturation level of 80% are shown in Fig. 6 [37]. More lipophilic compounds with higher distribution coefficients tended to exhibit comparatively higher S^M values. The highest values were observed for fenofibrate, attributable to excellent solubility in medium chain lipids and a rather dramatic loss of solubilization capacity in digested medium. The same

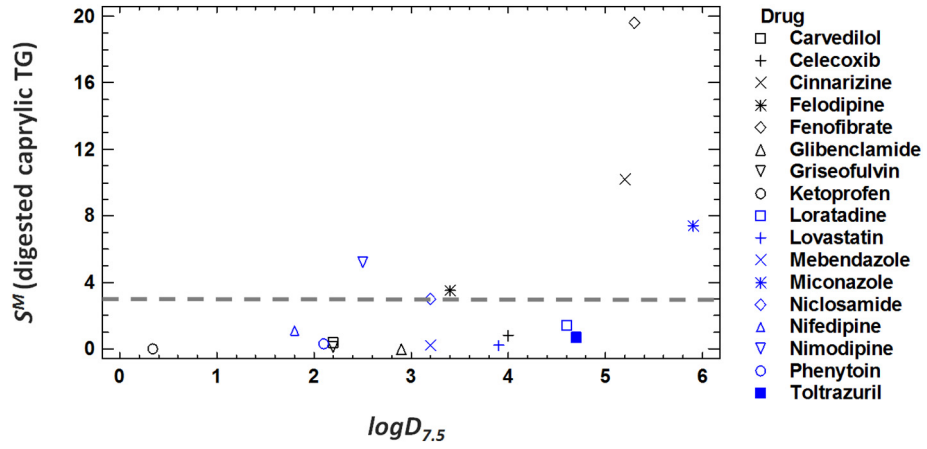

Fig. 6. Estimated maximum supersaturation, S^M, in the simulated digested aqueous phase based on caprylic triglyceride (TG). Different drugs are plotted as a function of their distribution coefficient at pH 7.5, log$D_{7.5}$. The dashed gray line marks an often-reported threshold value for S^M that represents a particular risk of drug precipitation *in vitro* at higher values (Figure adapted) [37].

comparison of S^M for the slightly longer chain capric triglycerides yielded reduced S^M values, and only fenofibrate was still above the arbitrary selected precipitation risk limit of $S^M > 3$ [37].

These findings suggest that S^M can be targeted by proper selection of the lipid formulation and dose. In the past, candidate LBFs were mostly selected for their ability to achieve maximum drug loading; however, such an exclusive focus on the undispersed formulation should be replaced by a more refined view in light of what we know today. Consideration of formulation solubility during the digestion phase, with the aim of achieving a balanced drug supersaturation that is maintained in the intestine is also encouraged.

Although simple parallel lipolysis testing can be used for an early screening of formulation candidates, several selected systems could be further studied in more sophisticated *in vitro* tests. Therefore, studies aiming at simpler or more sophisticated *in vitro* digestion tests complement each other as they apply to different phases of formulation development. Finally, a further trend for advancing *in vitro* lipolysis testing can be seen in research focused on real-time analysis. The goal is to study drug concentrations and structural changes in a dynamic way, which is meant to deliver valuable insights into the fate of the lipid-based delivery system. Availability of kinetic data opens the door for mathematical modeling that can become part of physiologically-based pharmacokinetic modeling, and initial research has already been published [66–68]. The combination of sophisticated analytics and mathematical modeling can help to approach topics that are hard to study experimentally; for example, understanding how the dynamic absorption of solubilizing excipients affects drug supersaturation *in vivo* [66]. Especially, the dynamic absorption of bile salts from solubilizing mixed colloids is expected to cause transient drug supersaturation close to the intestinal membrane [69–70]. It should not be forgotten that events in the intestinal milieu are far away from any thermodynamic equilibrium; therefore, dynamic changes must be considered for a better understanding and more realistic testing of LBFs. Hence, the following sections include a discussion of the first real-time analysis of concentration measurements followed by a monitoring of structural changes during dispersion and digestion of formulations.

4. Real-time analytics of drug supersaturation and precipitation during digestion *in vitro*

A scheme of classical *in vitro* lipolysis tests using a computer-controlled pH stat titrator system is depicted in Fig. 7. Details of this test, including medium composition, hydrodynamics, sampling, and applications have been excellently reviewed previously [71–72]; therefore, this article emphasizes different types of analytical monitoring. According to the nomenclature of process analytics, "off-line" indicates removal of samples that are analyzed with a significant time delay, usually in a separate laboratory. This is the case for classical sampling during lipolysis testing, as ultracentrifugation methods have been applied for an initial separation of phases. This type of sample preparation results in a solid phase of mostly calcium soaps with eventually precipitated enzyme(s) as well as drug and there is a further aqueous colloidal phase with solubilized drug, which can be accompanied by a residual oil phase of solubilized compound [71–72]. A subsequent quantification of drug *via* high performance liquid chromatography (HPLC) is part of an analytical procedure that is far from real-time conditions; therefore, it does not allow close monitoring of dynamic changes. Less of a time-delay is associated with the notion of "at-line" analytics, which are not synchronized with the process dynamics but typically involve little sample preparation and happen in proximity of the process (or *in vivo* test). Practically no time delay is given when samples are automatically drawn from the process for a separate real-time analysis. This is called "on-line" analytics and typically is based on a flow-through cell. Finally, the so-called "In-line" analytics makes use of an immersion probe for direct real-time analysis.

An in-line technique that measures free drug concentrations to determine true supersaturation instead of just apparent values is of interest as discussed before. An ion-selective electrode (ISE) was recently introduced to study free drug concentrations in colloidal systems by taking lipolysis into account [73]. Electrodes that are ion-selective were evaluated a few years ago for dissolution testing [74], including

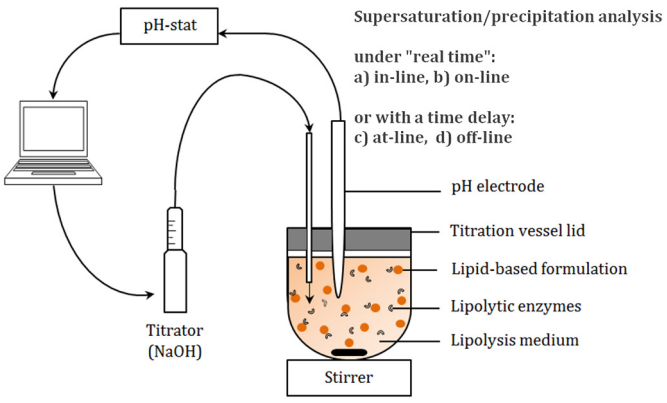

Fig. 7. Schematic representation of *in vitro* lipolysis with a computer-controlled pH stat titrator system. Real-time analyses are considered "in-line" if they use immersion probes or "on-line" if they make use of a flow-through cell. "At-line" analyses include those that analyze drawn samples with a small time delay close to the *in vitro* test. This is differentiated from an "off-line" mode for which results come from a separate analytical laboratory with a notable time delay to the timing of the *in vitro* test.

tests in biorelevant media [75]. Studies using these probes raised interest in overcoming limitations associated with standard in-line UV analytics that are based on fiber optical probes in rather turbid media. Such UV in-line probes are widely used in quality control testing; however, wavelength-dependent scattering of particles and colloids must be corrected by mathematical approaches such as multivariate analysis or second-derivative algorithms [76–77]. There are limits for such corrections in optically dense colloidal systems, and even though in-line UV has been used to analyze drugs in the presence of lipid-based excipients [78], the ISE should be more versatile in turbid media.

Potentiometric probes have to first be conditioned for a given charged analyte. As such, the ISE is soaked for several hours in a buffer containing the highest drug concentration to be analyzed, and fresh calibration is needed within less than a day prior to measurement [73]. Hydrochlorides of diphenhydramine and loperamide (LOP) were selected as SNEDDS compounds in a lipolysis study [73]. Results showed a rapid decrease in free drug concentrations following the onset of lipolysis, attributable to partitioning with the evolving colloidal species; drug precipitation and ion-pair formation with released fatty acids were discussed as possible causes of this decrease in concentration, as depicted in Fig. 8 for LOP. Changes in the drug distribution coefficient of dispersed *vs.* digested formulations are particularly expected to dominate free concentrations of lipophilic drugs. A drawback associated with using ISEs is the ability to only measure ionizable drugs that constitute only a portion of marketed compounds. These compounds with ionizable moieties are then mostly formulated as tablets; therefore, they just occasionally make good candidates for LBFs [5,79].

Development of new spectroscopy protocols for more versatile real-time analytics during lipolysis would need to manage the aforementioned turbidity, the complexity of medium, the dynamic changes over time, and low analyte concentrations. An in-line dispersive Raman spectroscopy method [80] has been successfully introduced that uses a multi-fiber probe (PhAT) based on a strong laser (400 mW) at a wavelength of 785 nm, as shown in Fig. 9. An advantage of this approach is that drug in solution can be quantified and distinguished from precipitated compound. However, this requires extensive calibration work from reference spectra preprocessed using different algorithms to remove sources of non-linearity and spectral information that are not attributable to drug concentration. These raw data are then used for a partial least-squares (PLS) regression model, in which different spectral ranges are evaluated for model inclusion. Fig. 9 outlines these different steps schematically to obtain the time-resolved concentration of precipitated drug (C_{pr}) or the concentration of solubilized compound. This approach has the advantage of a continuous real-time analysis and addresses issues regarding accuracy of the ultracentrifugation method when studying early time points of digestion [46,80]. The real-time analysis enables drug solubility to be modeled and demonstrates lipolysis-triggered apparent supersaturation. The following precipitation was then simulated using a nucleation and growth model [80]. Because of the good predictions obtained using fenofibrate as a model drug, a subsequent study used a combination of Raman spectroscopy and modeling to consider an absorption sink during a classical *in vitro* lipolysis study [66]. The basic idea is that once the modeling constants are determined in the non-sink digestion test *in vitro,* the results can be used for an extended model that considers drug absorption as well as the influence of solubilizing lipids on apparent supersaturation during absorption. This extended model adequately simulated *in vivo* profiles of lipid-based systems of fenofibrate in pigs [66]. Modeling is insightful; for example, it demonstrates how absorption in the intestine lowered the apparent supersaturation of fenofibrate, which was clearly below the values under the non-sink lipolysis condition [46,66].

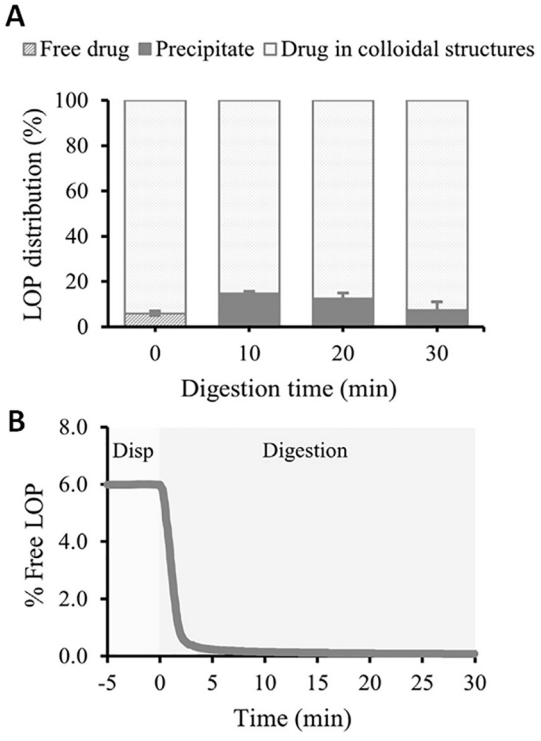

Fig. 8. *In vitro* lipolysis of nano-sized self-emulsifying drug delivery systems (SNEDDS) containing loperamide (LOP, 20 mg/g). A) Drug distribution in the different phases of *in vitro* samples following centrifugation is shown. B) Percentage of free drug during lipolysis measured by an ion-selective electrode is shown. "Disp" signifies the dispersion phase; further details are given in the text and in the original research article (Figure adapted) [73].

Use of a high energy radiation source, *i.e.*, a synchrotron beam, is another method used to manage analytical challenges during lipolysis. Such synchrotron small-angle X-ray scattering (sSAXS) has been used for real-time analysis of fenofibrate precipitation as well as to determine the solid form [81]. A peristaltic pump conveys samples from the *in vitro* lipolysis experiment into a flow-through capillary where the synchrotron X-ray beam passes the sample and transmits to the SAXS detector. Increase of scattering intensity suggested increased precipitation of fenofibrate that appeared in the thermodynamic most stable form. Fig. 10 shows the sSAXS profiles for fenofibrate as a SNEDDS model; the initial fenofibrate diffraction peaks were detected 4 min after adding the lipase. The time course is well represented by the peak at a q of 1.15 $Å^{-1}$, as shown in Fig. 10. The authors compared their results with polarized light microscopy of the pellet phase, and increasing fenofibrate crystals were observed in line with the real-time analysis using sSAXS [81].

Another study reported precipitation of a fenofibrate LBF using synchrotron X-ray scattering [82]. Real-time analysis of the *in vitro* drug crystallization was obtained in the wide-angle range (sWAXS) where the intensity of the radiation source enhanced sensitivity of crystalline particle detection compared to a standard WAXS. On the other hand, sSAXS provided rather information regarding the structural formulation changes during digestion. The real-time analysis of structural and hence colloidal changes is discussed in more detail in the following section; it is most valuable to combine real-time analyses during digestion. Indeed, the sSAXS experiments showed that SEDDS formulations of Kolliphor EL or Labrasol formed lamellar phase structures during digestion; however, when monoacyl lecithin was added to both drugs, formation of the lamellar structures was inhibited. Precipitation of fenofibrate differed for the four formulations (with and without monoacyl lecithin) during the last 45 min of *in vitro* digestion [82]; however, such differences were not observed in a rat pharmacokinetic study. As previously mentioned, *in vitro* discrimination of formulations does not necessarily translate into formulation differences *in vivo*.

A disadvantage associated with the wide applicability of sSAXS and sWAXS is that the methods are quite sophisticated, with limitations of experimental access to a synchrotron radiation source. Other methods

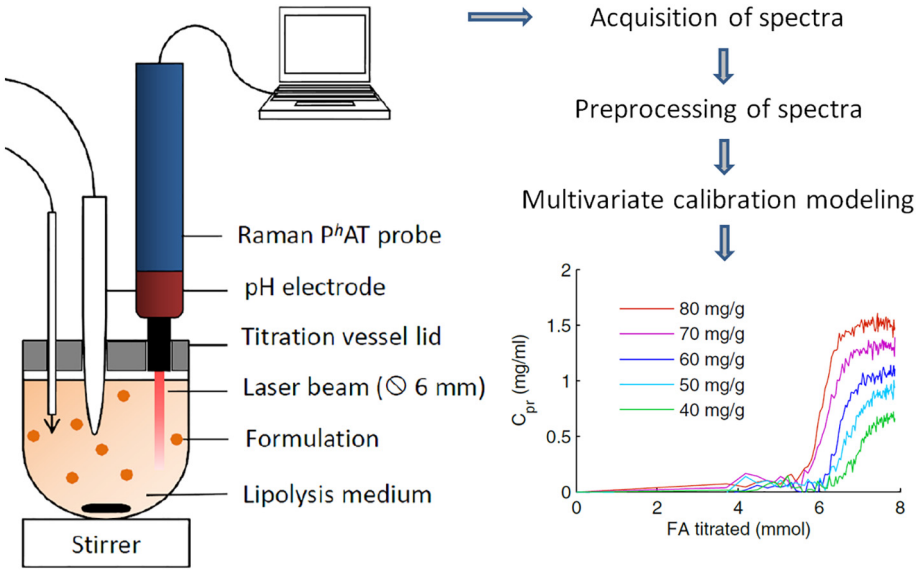

Fig. 9. *In vitro* lipolysis test equipped with a multi-fiber Raman probe (PhAT). The process flow is shown from data acquisition and preprocessing of the spectra to generation of a multivariate model. Precipitated drug concentration, C_{pr}, is depicted as a function of fatty acids (FA) titrated during lipolysis [80].

come with some analytical limitations, as previously discussed using ISE as an example, as it is not applicable for neutral molecules. Electron spin resonance (ESR) spectroscopy has an even smaller application scope, as drugs are rarely paramagnetic, which necessitates the use of spin probes. TEMPOL benzoate (TB) has been reported as a lipophilic model compound that has been used to study drug release during digestion of SEDDS [67]. Such release analyses are based on changes in ESR spectra that are caused by altered polarity of the medium surrounding the spin probe; however, changes in viscosity also affect spectroscopic results [83]. The hyperfine coupling constant in ESR spectra can differentiate surrounding media, which begins as an oil and eventually becomes mixed micelles or buffer solution at the end of drug release from a digested LBF [84]. Of course, structural changes in the surrounding drug medium directly affect solubility and supersaturation, so it is interesting to focus real-time analytics on how the structure is altered during lipolysis of an LBF.

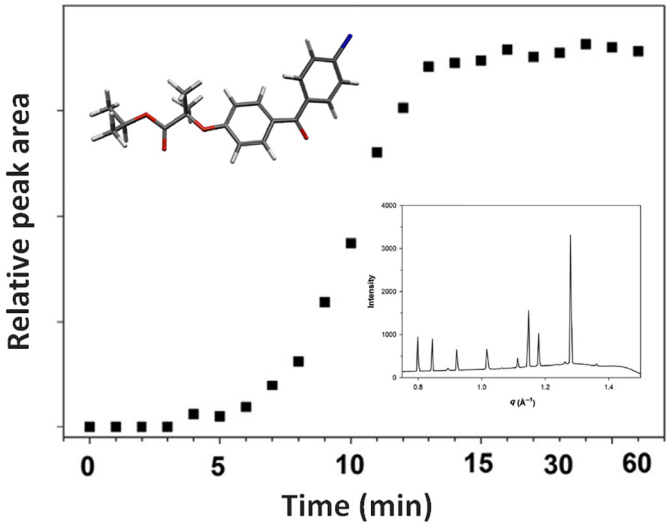

Fig. 10. Results of a synchrotron small-angle X-ray scattering (SAXS) study during *in vitro* lipolysis. Crystalline fenofibrate was detected 4 min after adding lipase, and kinetics of precipitation were further monitored *via* changes in a diffraction peak ($q = 1.15$ Å^{-1}) over time. Further details can be inferred from the original research work (Figure adapted) [81].

5. Approaches for monitoring structural changes during LBF digestion

Formulation digestion is a complex process, which starts already in the stomach as catalyzed by the gastric lipase and for the following intestine; there is not only the pancreatic lipase but also further lipolytic enzymes, which catalyze lipolysis. Pancreatic carboxyl ester hydrolase and pancreatic lipase-related protein 2 have been shown to play a dominant role in digestion of lipid excipients containing acylglycerols and polyethylene glycol esters [85]. Early work examining events on a structural level was based on freeze-fracture electron microscopy, which identified multilamellar and vesicular colloids as products of triglyceride hydrolysis [86]. Later work mostly used cryogenic transmission electron microscopy (Cryo-TEM) to study changes in lipid processing, with the advantage that samples were not fixed on a sample grid to avoid artifacts [87–88]. Particularly interesting was a subsequent study examining aspirates 30 min after administration of a heterogeneous liquid meal into the antrum; Cryo-TEM and atomic force microscopy (AFM) were used to study the structures of individual aspirates or aspirate supernatants following ultracentrifugation [89]. Such *ex vivo* studies are off-line from the viewpoint of process analytics, and structural information should be compared to other real-time analysis. Thus, an interesting real-time imaging of lipolysis was achieved using coherent anti-Stokes Raman scattering (CARS), which is a non-invasive technique with submicrometer resolution and millimolar sensitivity [90]. The information showing local chemical composition and phase behavior inside the oil droplets was a great analytical step forward and even though the resolution was not high enough for individual molecules, CARS was still adequate for monitoring drug partitioning from the excipient into the lipolytic products.

The powerful sSAXS technique for real-time analysis of lipolysis was previously mentioned (see section 4) and was initially used to study structural changes of simple digestible and non-digestible LBFs [91–92]. It is notable to compare off-line analyses following enzyme inhibition, which influences structural aspects of the digestion medium when compared to real time evaluations [92]. Indeed, these findings suggest that real-time analyses matter, at least when analyzing structural changes. Recent work using sSAXS investigated compositional effects in solid SMEDDS [93] using mixtures of digestible as well as non-digestible surfactants. Depending on the composition and presence of non-digestible surfactant, a significant lag time was evident. An increase in the fraction of digestible surfactant *versus* lipid ratio enabled

digestion with a reduced lag time; however, the time delay vanished when non-digestible surfactants were entirely substituted with digestible surfactants [93].

To better elucidate colloidal changes, it is beneficial to combine different real-time analytics. At the Hebrew University of Jerusalem, lipid mixtures or glycerin monooleate and tricaprylin were studied with lipase using SAXS together with attenuated total reflection Fourier transform infrared spectroscopy (ATR FT-IR) [94]. An inverse hexagonal mesophase formed when the LBF was placed in water that initially and gradually shrunk during enzymatic hydrolysis; a second kinetic stage was observed during which the mesophase gradually disintegrated. These different kinetic phases were followed using the lattice parameter from SAXS as well as the time evolution of hydroxyl vibrational bands, which suggested more than one kinetic regime [94]. Different kinetic phases are often observed during real-time analysis of LBFs. Our aforementioned in-line Raman study of lipolysis also showed two distinct kinetic phases [80]. The first phase was here rather quick compared to the following kinetics and this initial regimen was attributed to the first ester hydrolysis from the triglycerides.

Another interesting technique for monitoring of formulation digestion is small angle neutron scattering (SANS) although it is not actually a real-time analysis. This analytical approach is particularly suited to study changes in the colloidal domain. A collaboration of the Northeastern University in Boston and Massachusetts Institute of Technology (MIT) started with the hypothesis that SANS may reveal size and shape differences of micelles before and during *in vitro* simulated digestion [95]. Triolein was used as a model lipid, and the pre-lipolysis mixture of bile salts and phospholipids displayed micelles with an average volume of 40 nm^3 that increased 2.5-fold over a 2-h lipolysis experiment, attributable to insertion of monoglycerides and fatty acids [95]. The latter lipolysis products were also in the focus of another study based on neutron scattering and sSAXS [96]. Obtained scattering data indicated transitions in the size and shape of colloids. This was accompanied by a transfer of caprylic acid from the core of the micelles to the shell or into the bulk water upon increasing pH. Such research helps to better understand the structure of colloids that are formed by endogenous components and degradation products of a LBF during digestion.

Compared to rather sophisticated techniques like SANS, the most widespread analysis method of colloidal size is dynamic laser light scattering (DLS), which is widely used to characterize the type of dispersion obtained at high dilutions [12]. DLS is also helpful to determine the critical dilution at which a dispersed oil-in-water state is formed [97]. Even though modern DLS instruments can manage some multiple light scattering, the turbid lipolysis medium of classical *in vitro* tests is usually beyond the limits of adequate measurements. However, diffusing wave spectroscopy (DWS) is a complementary light-scattering technique for turbid systems. This method has been used to monitor emulsion changes *via* a decay time of the correlation function and the transport mean free path of photon scattering [98]. The same type of monitoring is promising for studies of LBF lipolysis; however, this has not yet been done. To date, DWS has been used with SEDDS to study rheology regarding capsule filling in a broad frequency range [99]. In addition, there is a digestion study of emulsions for which DWS results were compared to those from NMR [100]. The DWS data were evaluated for time-resolved diffusion, enabling an interesting comparison with NMR diffusion data. The latter results allowed monitoring of molecular evolutions, and thus, the events related to lipolysis and phase changes. In complement, the DWS results followed supramolecular changes (*i.e.*, colloids as they change in the course of lipolysis) in which it was possible to see the transition from vesicles to micelles. This was not the first work to employ NMR diffusivity data to study drug lipolysis; previously, different NMR methods were compared to evaluate phase changes of model systems during lipolysis [101].

To conclude the discussion on promising real-time techniques, the focused beam reflectance measurement (FBRM) technique is worth mentioning, which involves an in-line probe with a circulating laser that measures a cord length distribution of particles. FBRM was used to study drug precipitation during dilution of LBFs [50,102] and also has potential for digestion monitoring. However, different particle populations typically co-exist from droplets of the formulation to drug crystals and precipitated fatty acids, which presents a challenge. It remains to be explored how useful FBRM can be for detecting structural lipolysis-triggered changes and/or precipitation; this analytical situation can be compared to a completely different technique based on Taylor dispersion analysis combined with detection of fluorescence [103]. Recently, this particle sizing technique was successfully used to follow droplet size changes of dispersed SEDDS in the presence of lipase [104]. While Taylor dispersion analysis can analyze small colloidal particles, this is not possible with FBRM, however, the latter technique is truly an in-line method. In contrast, Taylor dispersion analysis requires drawing samples with minimal preparation; therefore, it might be considered to be at-line analytics.

The different measurement principles and probe techniques discussed here offer a wide spectrum of process analytics during lipolysis testing; however, no single technique currently fits all needs.

6. Concluding remarks and outlook

Over time, lipid-based vehicles, and SEDDS in particular, have shown potential to enhance oral absorption and reduce variability of exposure resulting from either erratic absorption in the fasted state or from food effects on oral bioavailability. Different mechanisms are known to play a role in drug absorption from SEDDS or other LBFs; however, a predominant factor is whether a formulation can sustain drug in solubilized form. Early *in vitro* studies emphasized solubilization; however, recent research has shifted the interest to drug supersaturation, as it is triggered by dispersion and/or lipolysis of lipids. Differences between apparent and true drug supersaturation were discussed, and a key conclusion is that different approaches are viable depending on their objective for using and interpreting data. Apparent supersaturation can be a good marker for drug precipitation, which often occurs at high concentrations in colloidal or droplet structures. However, it eventually becomes critical to follow the kinetics of apparent supersaturation for mechanistic modeling of drug transport at a liquid-liquid phase separation. Some modeling approaches have empirical constants that can accommodate a partition step of drug from colloids to the bulk phase, enabling data regarding apparent supersaturation to be useful again.

The discussion of drug supersaturation was complemented with a review of the different analytical measurement methods that are available. To truly capture the dynamics, real-time analytics for which different approaches exist with alternative levels of sophistication are preferred. Ion-selective electrodes or even in-line UV or Raman spectroscopy are methods that can be of interest for pharmaceutical development, even though the research character of the approach prevails. This emphasis on research is even more pronounced for synchrotron-based methods that are beyond every day laboratory analytics. However, they are widely applicable, and apart from precipitation kinetics, plenty of information can be obtained from SAXS and SANS regarding structural changes in the colloidal domain. In turn, these changes affect drug supersaturation kinetics, and such knowledge is important to design future LBFs in a more rational way. This is not only about lipids and surfactants but also holds for selection of polymeric precipitation inhibitors to sustain supersaturation, which is common practice in solid dispersions but has been studied more systematically during digestion of LBF only recently [61]. A more educated design of LBF would also profit from a better understanding of how individual drug properties cause supersaturation and the risk of precipitation. The previously mentioned small-scale solubilization experiments in simulated digested lipids [37] can be helpful to gain large amounts of data that would complement other more sophisticated experiments to target an

improved mechanistic understanding. A very recent article has pioneered to study the role of physico-chemical drug properties in lipolysis-triggered drug supersaturation and precipitation [105]. More of such research should be conducted to draw firm conclusions about how drug properties cause supersaturation in LBF and how the risk of precipitation can be predicted confidently.

Real-time analytics are of particular value to avoid artifacts of sample preparation and provide the means for mechanistic modeling of lipid-based formulation processing. Currently available computer software for physiologically-based pharmacokinetic models does not capture the relevant formulation dispersion and digestion processes of LBFs. Pioneering mechanistic models exist in the LBF literature; however, they should be refined and implemented in commercially available software. The interplay of *in vitro* models and mathematical modeling is likely to become more important in the following years. An *in vitro* test does not have to include all kinds of physiological features if, for example, an absorption step can be theoretically modeled. Likewise, a mathematical model needs to be formed using a reliable source of input data for which experimental *in vitro* tests are needed, at least in the foreseeable future.

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