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Development and characterization of solid lipid-based formulations (sLBFs) of ritonavir utilizing a lipolysis and permeation assay

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Abstract

As a high number of active pharmaceutical ingredients (APIs) under development belong to BCS classes II and IV, the need for improving bioavailability is critical. A powerful approach is the use of lipid-based formulations (LBFs) that usually consist of a combination of liquid lipids, cosolvents, and surfactants. In this study, ritonavir loaded solid LBFs (sLBFs) were prepared using solid lipid excipients to investigate whether sLBFs are also capable of improving solubility and permeability. Additionally, the influence of polymeric precipitation inhibitors (PVP-VA and HPMC-AS) on lipolysis triggered supersaturation and precipitation was investigated. One step intestinal digestion and bicompartmental permeation studies using an artificial lecithin-in-dodecane (LiDo) membrane were performed for each formulation. All formulations presented significantly higher solubility (5 to >20-fold higher) during lipolysis and permeation studies compared to pure ritonavir. In the combined lipolysis-permeation studies, the formulated ritonavir concentration increased 15-fold in the donor compartment and the flux increased up to 71 % as compared to non-formulated ritonavir. The formulation with the highest surfactant concentration showed significantly higher ritonavir solubility compared to the formulation with the highest amount of lipids. However, the precipitation rates were comparable. The addition of precipitation inhibitors did not influence the lipolytic process and showed no significant benefit over the initial formulations with regards to precipitation. While all tested sLBFs increased the permeation rate, no statistically significant difference was noted between the formulations regardless composition. To conclude, the different release profiles of the formulations were not correlated to the resulting flux through a permeation membrane, further supporting the importance of making use of combined lipolysis-permeation assays when exploring LBFs.

Keywords: solid lipid-based formulations, lipolysis, permeation, flux, solubilization, supersaturation, precipitation

Abbreviations:

4-BBBA	4-bromophenylboronic acid
ACN	Acetonitrile
AIDS	Acquired Immunodeficiency Syndrome
API	Active pharmaceutical ingredient
ANOVA	Analysis of variance
AUC	Area under the curve
BCS	Biopharmaceutics Classification System
DSC	Differential scanning calorimetry
DMSO	Dimethylsulfoxide
TPGS	D-α-tocopherol polyethylene glycol 1000 succinate
ENA	Enabling Absorption
FaSSIF	Fasted State Simulated Intestinal Fluid
FeSSIF	Fed State Simulated Intestinal Fluid
FaSSGF	Fasted State Stimulated Gastric Fluid
FFAs	Free fatty acids
HPLC	High pressure liquid chromatography
HLB	Hydrophilic lipophilic balance
HPMC-AS	Hydroxypropylmethylcellulose acetate succinate
IVIVC	In vitro-in vivo correlation
LiDo	Lecithin in Dodecane
LBF	Lipid based formulation
LY	Lucifer Yellow
PI	Precipitation Inhibitor
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
PVDF	Polyvinylidene fluoride
PVP-VA	Kollidon VA64
PVP	Poly(vinyl pyrrolidone)
sLBF	Solid Lipid-Based Formulation
UV	Ultraviolet
XRD	X-Ray Diffraction

1. Introduction

Approximately 40 % of drugs currently on market and up to 90 % of drugs in development show poor water solubility.[1-3] For drugs of BCS Class II and IV [4] it is necessary to develop and utilize enabling formulations to enhance bioavailability during pharmaceutical development.[5] A promising formulation strategy to increase bioavailability is the use of lipid-based formulations (LBFs) as the positive effect of ingested lipids on solubility and permeation of poorly soluble drugs has been well established.[6]

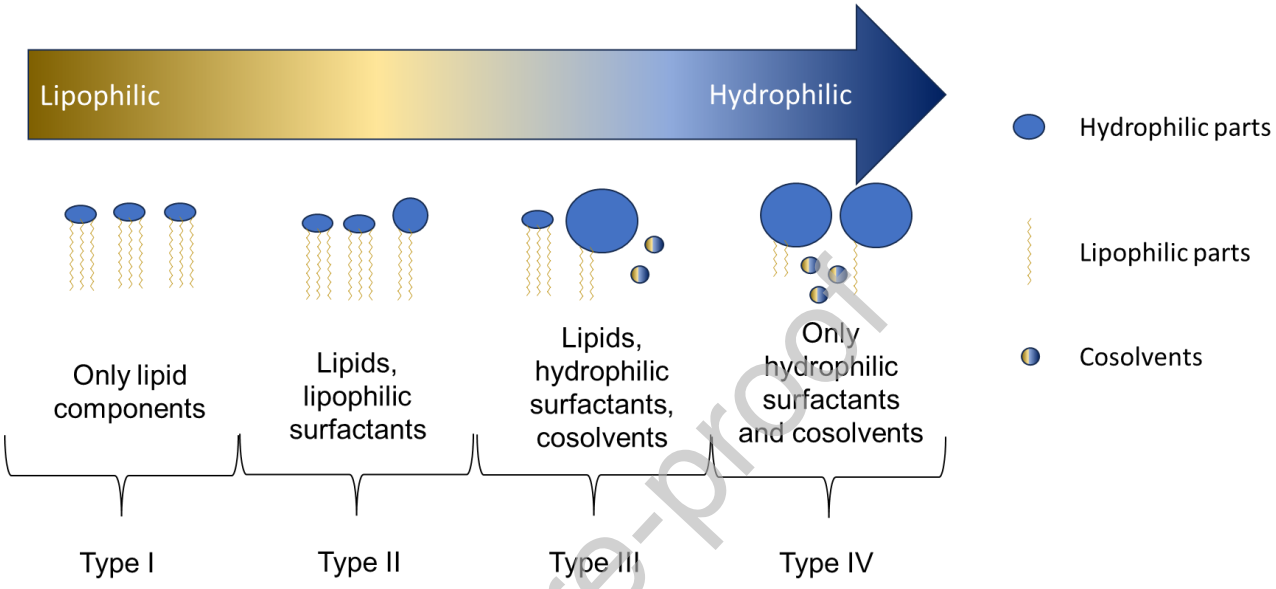

Figure 1. Visualization of the lipid-based formulation classification system.[7]

According to the lipid formulation classification system, formulations are classified from Type I to IV (Figure 1).[7] While Type I formulations consist only of a lipid component like triglycerides and the drug, type II formulations additionally contain water-insoluble surfactants, which increase the solubility of the drug and aid in emulsification. In Type III formulations, lipid components are combined with surfactants and cosolvents to further increase the solubility of the active pharmaceutical ingredient (API) in the small intestine and support spontaneous self-emulsification during digestion.[6-8] Type IV formulations only contain water soluble surfactants and cosolvents that can increase drug solubility in the formulation considerably, but no lipid components. Downsides of high surfactant and cosolvent concentrations are potential drug precipitation when diluted in the intestinal fluids as well as risk of irritation in the intestine in combination with poor tolerance[8-10]. Most LBFs are liquid in the form of oral solutions or solid soft capsules filled with liquids.[11-13] Limitations of these formulations include for example the instability of the lipid components, less stable APIs in solution than in solid forms, leakages from capsules, and risk of drug precipitation in the formulation as such due to cosolvent evaporation.[13, 14] To overcome these disadvantages, the interest in solid LBFs (sLBFs) has increased.[13, 15] sLBFs can be produced *via* spray drying, melt granulation, extrusion/spheronization, and adsorption to solid carriers.[9, 13] Most of these techniques rely on a chemically inert solid carrier on which liquid LBFs are adsorbed in an additional production step,[13] leading to a more complicated work flow with

reduced drug loading capacity and less economic feasibility. Solvent free melting techniques offer an opportunity to realize higher drug loads without intricate work flow. Several studies demonstrated that solvent free melting techniques are a simple approach to produce sLBFs with certain limitations regarding to the polymorphic form.[16-19] Thus, most of studies about solvent free melting techniques focus on taste masking and controlled release, but not on lipolysis and permeation[20].

While the influence of lipolysis on performance of liquid LBFs (including permeation, flux and/or absorption) is well described in literature, such studies have not been executed for solid lipid excipients and sLBFs.

The need for prediction of *in vivo* intestinal permeation behavior based on *in vitro* data is also crucial in drug development. Consequently, data from *in vitro* lipolysis and permeation assays are highly valuable for formulation design of LBFs.[21, 22] Lipolysis studies may be performed as simple one step experiments to evaluate the digestion behavior of the lipid components and solubility enhancement in the small intestine.[23-26] Permeation studies are used to understand how improved solubility may lead to higher permeation and hopefully higher bioavailability. The authors have previously established and reported methods for *in vitro* lipolysis and permeation studies for solid LBFs.[27]

In the present study, two class III solid LBFs with different lipid/surfactant ratios were manufactured using the solvent free melting technique to investigate the influence of triglyceride and surfactant concentrations on lipolysis and permeability. The aim was to investigate if higher triglyceride concentrations may reduce the API precipitation during lipolysis. Ritonavir (BCS Class IV [28]), which is a well-known pharmacokinetic booster for reverse transcriptase inhibitors in Acquired Immunodeficiency Syndrome (AIDS) therapy, was used as model drug. Ritonavir was selected as model drug for the investigation of sLBFs based on its low reported water solubility of 1 µg/ml[29], its comparably low melting point of 129.5 °C (own data) and its logP of 3.9-5.6[30, 31]. The influence of formulations on lipolysis and permeation behavior of the drug was investigated using the Enabling Absorption (ENA) device, which allows simultaneous monitoring of lipolysis and permeation.[22] An additional aim of this study was to investigate whether precipitation inhibitors can increase the permeation rate, as previous studies have reported the beneficial effect of such materials on supersaturated formulations.[32-36]

2. Materials and Methods

2.1 Materials

Hard fat (Witepsol® E 85) and glyceryl monostearate (IMWITOR® 491) were kindly donated by IOI Oleo GmbH (Hamburg, Germany). Polyethylene glycol monostearate I (PEG-monostearate, Gelucire® 48/16) was provided by Gattefossé (Saint-Priest, France). Ritonavir (> 99%) was purchased from Shanghai Desano Pharmaceuticals Co., Ltd. (Shanghai, China). 1-Vinyl-2-pyrrolidone-vinyl-acetate copolymer (Kollidon® VA 64) was kindly donated by BASF (Ludwigshafen, Germany). Hydroxypropyl methylcellulose acetate succinate (HPMC AS, AQOAT® AS MMP) was purchased from Shinetsu

(Tokio, Japan). Sodium chloride (NaCl), sodium hydroxide (NaOH) pellets, calcium chloride ($CaCl_2$) granules, phosphate buffered saline (PBS) tablets, Trizma® maleate (TRIS maleate salt), D-α-tocopherol polyethylene glycol 1000 succinate (TPGS), 4-bromophenylboronic acid (4-BBBA, ≥ 95.0 %), pancreatin from porcine pancreas (8 x USP specifications), Acetonitril (ACN, ≥99.9 %) and dimethyl sulfoxide (DMSO, ≥ 99.9 %) were obtained from Merck (Darmstadt, Germany). Lecithin (L-α-phosphatidylcholine) soy phosphatidylcholine extract (20 %) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Ethanol (99.5 %, denatured with isopropyl alcohol) was purchased from Solveco (Rosenberg, Sweden) and n-dodecane (≥99 %) was obtained from Alfa Aesar (Lancashire, UK). Lucifer yellow (LY) CH dilithium salt and FaSSIF/FeSSIF/FaSSGF powder were obtained from Biotium (Fremont, CA, USA) and biorelevant.com (London, UK), respectively. For all experiments, Ultrapure Milli-Q® water (grade I) from a direct water purification system (Merck, Darmstadt, Germany) was used. The chemical composition and melting points of the main ingredients of the sLBFs are shown in Table 1.

Table 1. Chemical composition and melting point of lipid excipients used.

Excipient Name	Witepsol© E85	Imwitor© 491	Gelucire© 48/16
Chemical Description	Hard fat; mixture of mainly saturated mono-, di- (~ 10 %), and triglycerides (~ 90 %) ; Fatty acid composition is C12: 30-35 %, C14: 10-15 %, C16: 20-25 %, C18: 22-28 %	Glyceryl monostearate	PEG-32 esters of palmitic and stearic acid
Melting point [°C]	43	66 - 77	46 - 50

2.2 Preparation of solid lipid-based formulations

sLBFs were prepared using Witepsol® E 85, Gelucire® 48/16 and Imwitor® 491. Two different mixtures were prepared from these excipients (sLBF1 and sLBF2). Precipitation inhibitors were also added to the mixtures (sLBFs 1a/1b and 2a/2b). The composition of each mixture is shown in Table 2. The following three steps were carried out for the preparation of the lipid based formulations: 1) the components were accurately weighed and stirred using a magnetic stirrer (MR Hei Standard, Heidolph Instruments GmbH & Co., Schwabach Germany) at 130 °C, 250 rpm for 15 min. 2) after cooling for two hours at room temperature, the LBFs were further cooled with liquid nitrogen and milled using a AICOK TB138M (Aicok Home Essentials) food processor. 3) after milling, the formulations were sieved (AS 200 Control, Retsch GmbH, Germany) at 1.5 mm amplitude for 10 minutes. The size fraction from 315 to 500 µm was used for further studies. Precipitation inhibitors were weighed and mixed with the formulations prior to experiments.

Table 2. Composition of the solid lipid based formulations.

Formulation Name	WITEPSOL® E 85 [%]	Gelucire® 48/16 [%]	IMWITOR® 491 [%]	Kollidon® VA 64 [%]	AQOAT® AS-MMP [%]	Ritonavir [%]
sLBF 1	45.0	45.0	-	-	-	10.0
sLBF 1a	40.5	40.5	-	10.0		9.0
sLBF 1b	40.5	40.5	-	-	10.0	9.0
sLBF 2	60.0	25.0	5.0	-	-	10.0
sLBF 2a	54.0	22.5	4.5	10.0	-	9.0
sLBF 2b	54.0	22.5	4.5	-	10.0	9.0

2.3 Characterization of solid lipid-based formulations

To determine the particle size distribution of sLBFs laser diffraction (Mastersizer 3000, Aero S Dispersion Unit, Malvern Panalytical GmbH, Kassel Germany) was used at 3 bar dispersion pressure. The Fraunhofer theory was used for data evaluation.

DSC measurements were performed with a heating rate of 10 K/min in a range of -10 °C to 200 °C (DSC 1, Mettler-Toledo AG, Switzerland). Heating was performed in two cycles, with a first heating to 200 °C followed by a cooling back to -10 °C and a second heating to 200 °C. For the evaluation, data from the first heating cycle was used to investigate the influence of the manufacturing process on the solid state.

X-Ray Diffraction (XRD) measurements were carried out in single measurements of the formulations. The measurements were carried out using a MiniFlex® 300 (Rigaku, Tokyo, Japan) equipped with a Cu K-α-X-ray source at 15mA and 40kV. Step size was set at 1.7 °/min with an angular range of 2-50° 2θ. Pure ritonavir and excipients were also measured.

2.4 Buffer and media preparation

For the intestinal lipolysis studies, a fasted state simulated intestinal fluid (FaSSIF) medium containing 2 mM TRIS maleate, 1.4 mM $CaCl_2$, 150 mM NaCl and 2.24 g/l FaSSIF powder was used. After dissolving the buffer salts (NaCl, $CaCl_2$ and TRIS maleate), the FaSSIF powder was added and the medium was stirred for two hours. Thereafter, the pH was adjusted to 6.5.

The same medium with a higher buffering capacity (200 mM TRIS maleate) was also used in the donor compartment during lipolysis-permeation studies. During these studies, a high buffering capacity was needed as titration of the released FFAs is not possible in the presence of the artificial membrane.[24] For the receiver compartment, PBS with a concentration of 10 mM phosphate buffer, 2.7 mM potassium

chloride and 137 mM sodium chloride at pH of 7.4 was used. To efficiently extract ritonavir from the lecithine-in-dodecane absorption membrane, 0.2 % w/v TPGS was added to the receiver medium.

2.5 *In vitro* intestinal lipolysis studies

In vitro lipolysis studies were performed in accordance with the protocol reported by Alskär *et al.*[37] The lipolysis setup is shown in Figure 2A. In each experiment, 1.25 g of sLBF under study was dispersed in 40.56 ml FaSSIF medium (pH 6.5) at 37 °C in the lipolysis vessel and stirred at ~ 450 rpm using a propeller stirrer. After 10 minutes of dispersion time, 4.44 ml pancreatin extract were added. The pancreatin extract was freshly prepared by dispersing 1.2 g pancreatin from porcine pancreas in 6 ml of cold lipolysis buffer followed by centrifugation at 5 °C and 3500 rpm (5810R, Eppendorf, Hamburg, Germany) for 15 minutes to separate the precipitate without inactivating the enzyme. During the entire lipolysis study, the pH was kept constant at 6.5 using a 907 Titrando Metrohm (Herisau, Switzerland) autotitration device with 0.6 M NaOH. A circulating water bath kept the temperature at 37 °C. Samples (1 mL) were withdrawn at predefined time points and mixed with 5 µl of 0.5 M 4-BBBA solution (in methanol) to inhibit enzymatic activity. After centrifugation at 21,000 g and 37 °C for 15 minutes, the aqueous phase was filtered using Minisart® RC25 syringe filters with pore size of 0.2 µm (Sartorius, Göttingen, Germany) since the centrifugation did not result in complete separation of oil, aqueous and solid phases. The filtrates were diluted in ACN (1 filtrate:9 ACN) and stored at – 18 °C until HPLC-UV analysis. The free fatty acids released were calculated based on the amount of titrated NaOH minus the amount of NaOH used for blank experiments. The unionized fraction of free fatty acids was calculated in the same way after back titration of the 60 minutes digestion sample. The percentage of the digested FAs was calculated based on the saponification values of the compositions of the formulations provided by the manufacturers, as the exact composition was not determinable.

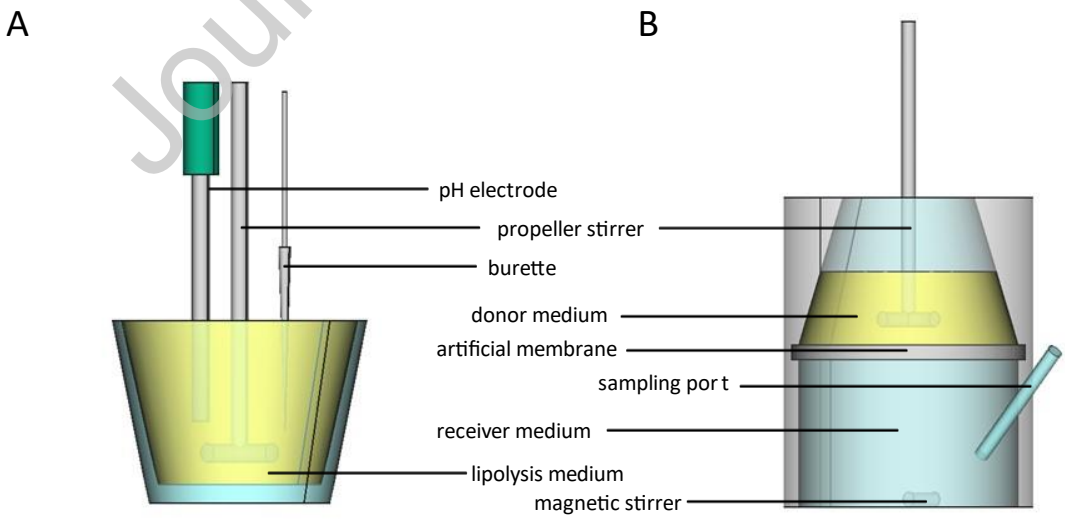

Figure 2. A: Setup of *in vitro* lipolysis studies. B: ENA device, setup of *in vitro* lipolysis permeation studies.

2.6 *In vitro* lipolysis-permeation study

2.6.1 Preparation of artificial membrane

For the *in vitro* lipolysis-permeation studies, an artificial lecithin-in-dodecane (LiDo) membrane was used as permeation membrane. The preparation of the membrane was performed according to Hedge *et al.*[24] Two grams of lecithin were dissolved in 10.0 ml of 1.5 % v/v ethanol in n-dodecane for ~ 5 h. To separate precipitated or undissolved material from the dissolved lecithin, the solution was centrifuged at 20 °C and 3,220 g for 20 min. After aliquoting, the supernatant was frozen at -18 °C and thawn at room temperature 30 minutes before the experiment commenced. To prepare the artificial membrane, 713 µl of LiDo solution were pipetted onto a polyvinylidene difluoride (PVDF) filter support (Immobilon®-P Transfer Membrane, 0.45 µm pore size, Millipore, Merck, Darmstadt, Germany) with an area of 44 cm^2; a translucent membrane indicated full adsorption of the LiDo to the PVDF support.

2.6.2 Lipolysis-permeation studies

Lipolysis-permeation studies were performed using the ENA device introduced by Keemink *et al.*[22] The setup of the ENA device is shown in Figure 2B. In the upper compartment of the device (donor compartment), the sLBF is dispersed and digested simulating the small intestine conditions.[38] The lower compartment (receiver compartment) simulates the blood and is separated from the donor compartment by the LiDo membrane. The receiver chamber was filled with ~235 ml of prewarmed (37 °C) PBS containing 0.2% w/v TPGS (pH 7.4). After heating to 37 °C, 54.08 ml FaSSIF medium (pH 6.5) with increased buffering capacity were transferred to the donor chamber. A stock solution of 10 mM Lucifer Yellow was added (60 µL) to the donor medium as membrane integrity marker. Thereafter, 1.5 g of the sLBF or, in case of studies using pure drug, 150 mg ritonavir, were added and dispersed for 10 min using a propeller mixer at ~ 450rpm. After the dispersion phase, 5.92 ml of freshly prepared pancreatin extract were added to the donor chamber to initiate the digestion. The pancreatin extract was produced by dispersing 1.6 g of pancreatin from porcine pancreas in 8.0 ml lipolysis buffer (5 °C), followed by centrifugation at 3500 rpm and 5 °C for 15 min, and collection of the aqueous phase. To determine the ritonavir concentration, samples from both chambers were taken during the experiment at 0, 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 min. For the donor samples, 750 µl were taken and enzymatic activity was inhibited by the addition of 3.75 µl of 0.5 M 4-BBBA, samples were then treated similarly as described in 2.5. The receiver samples (300 µl) were split in two. One part was used to detect LY concentration, the other for the determination of ritonavir concentration. LY samples were diluted 1:3 in ACN and transferred to a 96-well plate (Costar®, black flat bottom, Corningm Kennebnuk, ME, USA). The receiver samples and donor samples for drug quantification were stored at -18 °C until HPLC-UV analysis.

2.7 Sample Analysis

2.7.1 LY analysis

To investigate membrane integrity, LY was quantified using a Tecan Spark® (Männedorf, Switzerland) plate reader in fluorescence mode. The emission and excitation wavelengths were set to 536 nm and 428 nm, respectively.

2.7.2 HPLC-UV analysis of ritonavir

To determine the ritonavir concentration, a HPLC-UV system (1290 Infinity, Agilent Technologies, Santa Clara, USA) was used with a ZORBAX Eclipse XDB-C18 (4.6 x 100 mm, 3.5 µm) (Agilent Technologies, Santa Clara, CA, USA) column. An isocratic method at 30 °C with a mobile phase of 45 % MilliQ® water and 55 % ACN (v/v) at a flow rate of 0.75 ml/min was used. The detection wavelengths were set to 210 nm and 240 nm with an injection volume of 20 µl. The retention time was ~ 5.2 min. Calibration curves were linear (R^2> 0.999) within the range of 0.12 to 125 µg/ml.

2.8 Data analysis

Unless stated otherwise, all experiments were performed in triplicates. Data are presented either as mean ± standard deviation or single measurements. Statistical significance was tested by one-way ANOVA followed by a Fisher's least significant difference test to distinguish which formulations showed differences amongst each other. Data evaluation and statistical analysis was done with Microsoft Excel, while data visualization was performed with OriginPro 2020 (OriginLab Corporation).

The permeations rates were calculated according to equation 1. The mean permeation rates were calculated with the arithmetic mean of all permeation rates from minute 45 and higher for each composition.

$$permeation\ rate\ [\frac{\mu g}{min}] = \frac{c(t) * V(t)}{t_i} \qquad \text{Equation 1}$$

Equation 1: calculation of permeation rates, c(t) = concentration (µg/ml) at timepoint t, V(t) = Volume (ml) of receiver medium at timepoint t, t_i = time interval (min) between sampling

3. Results and discussion

3.1 Composition of solid LBFs

Initially, different combinations of solid lipid excipients were spiked with 10 % of ritonavir, melted at 60 °C, and stirred at 25 rpm for 48 h. Solid state analysis of the different formulations *via* DSC revealed that sLBF 1 did not show any traces of crystalline ritonavir. Higher concentrations > 10 wt.-% ritonavir were also investigated but traces of crystalline material were detected in all cases. The other formulations were consequently designed based on sLBF1. sLBF 1a, 1b and sLBF 2, 2a, and 2b were developed to investigate how *in vitro* lipolysis and permeation is influenced by the ratio of lipid and surfactant as well as the presence of precipitation inhibitors. All developed sLBFs belong to class IIIA of the lipid formulation classification system introduced by Pouton *et al*.[7]

3.2 Particle size and solid state characterization of solid lipid-based formulations

After milling the particle size distributions of obtained sieve fractions were determined (Table 3). The graphs of these measurements can be found in the Supplementary Information (Figure S1). sLBF 2 has a wider particle size distribution and a larger mean particle size. This size difference may lead to higher variation in the lipolysis-permeation studies due to differences in the accessible surface area of the particles during digestion. Lipolysis will naturally be slower for formulations containing the same lipids when the particle size distribution is larger due to the lower total surface area exposed to the enzyme containing solution.

Table 3. Percentiles of particle size distribution of LBF 1 and LBF 2 after sieving (mean ± SD, n = 3)

	LBF 1	**LBF 2**
X_{10} (µm)	305 ± 2	311 ± 3
X_{50} (µm)	417 ± 3	449 ± 4
X_{90} (µm)	572 ± 5	648 ± 4

The results from the XRD measurements are shown in Figure 3. The physical mixture describes the physical blend of the substances, while the melted formulation is the produced sLBF. Preliminary studies of the pure excipients and ritonavir showed isolated ritonavir peaks between 8 to 12° 2θ (marked with red lines). sLBF 1 shows a minor peak at 8° 2θ shifted to a smaller angle, indicating that traces of ritonavir are still crystalline. This is in contrast to the results described in 3.1, where samples were heated to 60 °C for 48 hour. During the current experiments, a temperature of 130 °C was used but stirring lasted only 15 min. In any case, the small peaks indicate that the crystalline amount of ritonavir was strongly reduced. No peaks in this range are visible for sLBF 2. This suggests that the API is either amorphous or molecularly dispersed.[16, 19] Comparing both formulations, LBF 2 is likely more stable as crystalline traces may induce quicker recrystallisation in sLBF 1.[39] DSC data corroborating the XRD data is shown in the supplementary information (Figure S2).

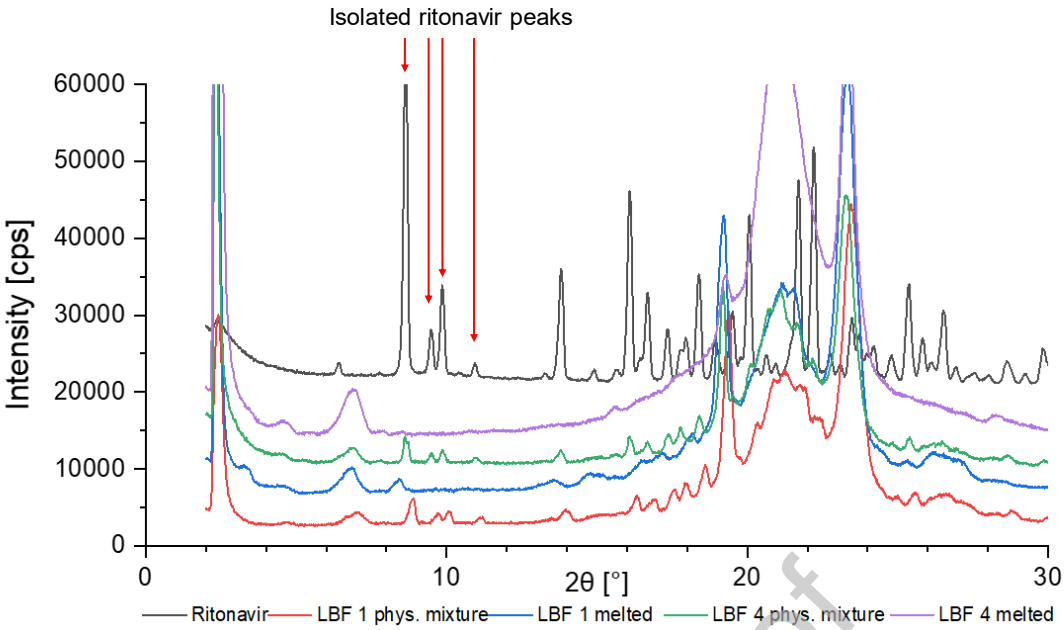

Figure 3. X-Ray diffraction spectra of pure ritonavir and sLBF 1 and 2 either as physical mixtures (red and green curves) or after melt preparation (blue and purple curves). Red lines indicate isolated ritonavir peaks.

3.3 *In vitro* lipolysis studies

3.3.1 Lipid digestion

The results of the lipid digestion studies mimicking lipolysis in the small intestine are shown as the amount of ionized free fatty acids (FFAs) released over time in Figures 4A and 4B. A zoomed version of Figure 4A to distinguish between the single curves may be found in Figure S3 in the Supplementary Information. The unionized FFAs are presented as total amounts in Figure 4C and 4D. When comparing the two sLBFs without PI, the total amount of digested ionized and unionized free fatty acids is higher for sLBF 2 (36.8 %) as LBF 1 (16.4 %). These results were expected as the amount of triglyceride (a highly digestible component) in sLBF 2 was 15 % greater compared to sLBF 1. At the beginning of the lipolysis (~ 4min) the amount of FFAs released is about 18-fold higher for sLBF 1 (31.7 ± 13.3 µmol) compared to sLBF 2 (1.71 ± 3.0 µmol). This may be explained by the improved wettability of sLBF 1 due to the higher amount of Gelucire 48/16, a water soluble surfactant with an HLB of 12[40] .

The biphasic curve of sLBF 2 may be explained by splitting the digestion process in two phases. In the first phase, the surfactant is mainly digested, since it dissolves faster than the triglyceride in the aqueous medium. Another reason for the increase in the first phase may be restructuring of the colloidal system as a result of the increased amount of released FFAs, which can also act as surfactants. In the second phase beginning after ~25 minutes, the digestion rate increases again as triglycerides are digested. sLBF 1 also presents a biphasic release of FFAs even though it is less pronounced. It is hypothesized that it takes longer until the higher amount of surfactant in the formulation is fully digested. A longer digestion

experiment of this formulation was already performed in an earlier study,[27] which showed a curve shape comparable to that of sLBF 2.

Formulations including PVP-VA as PI (sLBF 1a and 2a), show a tendency of slower digestion compared to sLBF 1 and 2. The HPMC-AS loaded formulations (sLBF 1b and 2b), on the other hand, behave similar. This may be explained by the acidic nature of HPMC-AS, which could lower the pH and falsify the digestion rate by overcompensating for the amount of NaOH consumed. The ionized and total amount of free fatty acids in formulations sLBF 2a (31.1 %) is reduced compared to sLBF 2, indicating that PVP-VA could inhibit the digestion in some way. Yet, this is not shown for sLBF 1 (19.1 %), which is surprising. One explanation could be that PVP-VA influences the digestion of Witepsol® E85 but not of Gelucire® 48/16, which is suspected to be primarily digested only after 60 minutes. This may be a reason that the effects in sLBF 1a is too small to be observable. The similar tendency is shown for sLBF 1b (10.21 %) compared to LBF1. Due to the high variations in sLBF 1, these formulations do not show any significant differences. No strong effect of PIs on digestion is discernible from the obtained data. It is likely that they do not interfere with enzymatic activity as differences are only observable for unionized and not ionized fatty acids. As digestion plays a major role in the effectivity of LBFs, additional experiment can be performed to identify potential benefits of PI addition.[10]

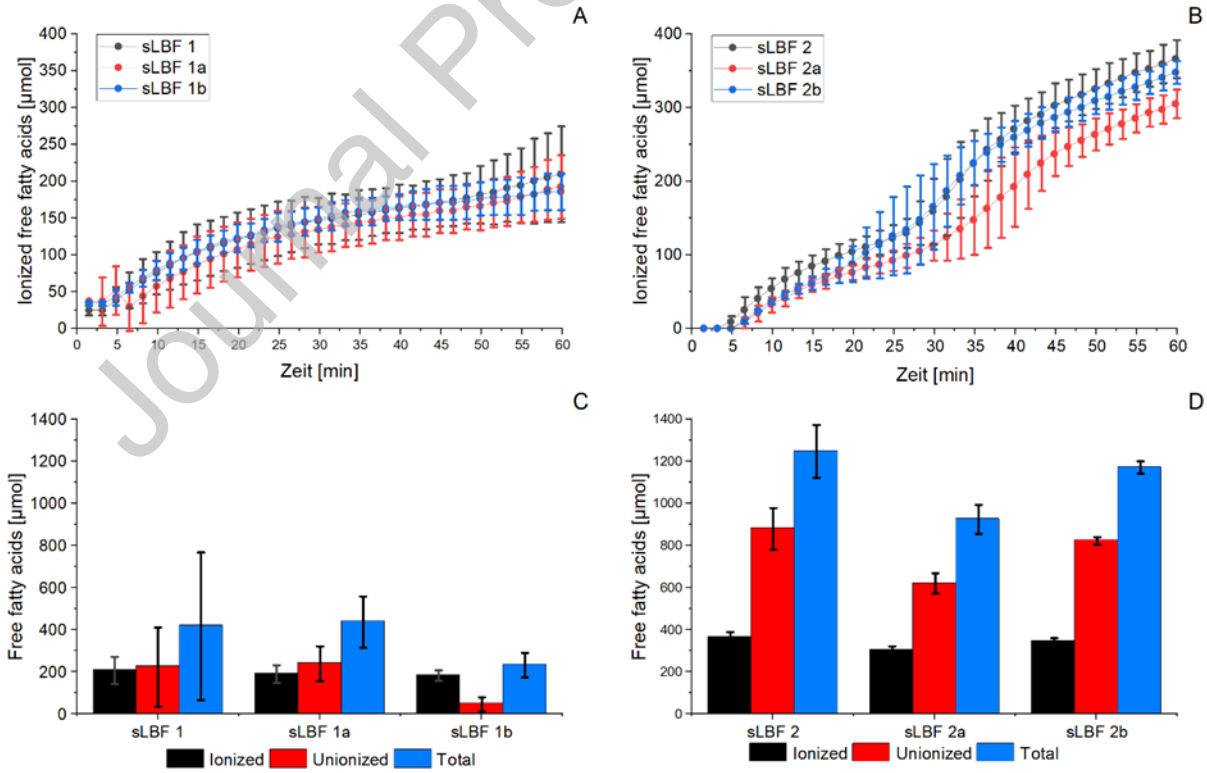

Figure 4. Released amount of ionized FFAs over time for LBF 1 to 1b (A) and LBF 2 to 2b (B) and total amount of free fatty acids released for LBF 1 to 1b (C) and LBF 2 to 2b (D). Data presented as mean ± SD (n=3).

3.3.2 Supersaturation of ritonavir during the lipolysis assay

The concentration of ritonavir during the lipolysis experiments is shown in Figure 5A. The data mostly reflect the typical curve shape of supersaturated solutions.[33, 41] During the dispersion time (-10 to 0 minutes), the concentration increases above the saturation concentration of ritonavir. After the addition of the pancreatic extract, ritonavir starts precipitating as indicated by the decrease of the concentration over time. As described above, we hypothesize that the better soluble surfactant is digested first after enzyme addition. This will in turn reduce the apparent drug solubility, which may trigger partial recrystallization and lead to the observed effect.[7, 8] While the ritonavir concentration is reduced by 76.8 µg/ml from 161.05 to 84.22 µg/ml for sLBF 1 within 60 min, the concentration for sLBF 2 decreases only by 48.3 µg/ml from 76.4 µg/ml to 28.1 µg/ml. The relative reduction of the ritonavir concentration in sLBF 2 is higher and sLBF 1 shows a significantly higher AUC compared to sLBF 2 (7212.97 [µg/ml]*min vs. 2650.33 [µg/ml]*min).

Liquid LBF Type III formulations with high amounts of water soluble components (> 40%) generally show higher precipitation rates.[7] Accordingly, a more pronounced precipitation was expected for sLBF 1 compared to sLBF 2. Surprisingly, precipitations of 48 % for sLBF 1 and of 73 % for sLBF2 were observed. The higher supersaturation ratio of sLBF 2 of 2.72 compared to the one of sLBF 1 of 1.91 may be the reason for this unexpected difference. The risk of precipitation is more pronounced with higher supersaturation ratios. The supersaturation ratio is calculated with the drug concentration after 60 min. As the concentration of ritonavir in sLBF 1 is still slightly decreasing after 60 min, the super saturation ratio may be underestimated still.

The addition of polymers as PIs to the sLBFs did not significantly influence the precipitation of dissolved ritonavir. Both polymers led to comparable AUCs and differences were non-significant. Several studies showed that polymers like HPMC and PVP-VA can prolong the precipitation of ritonavir from amorphous solid dispersions[32, 41] and LBFs.[42] The lack of effect was, therefore, surprising. A major difference in our study compared to literature is that the polymers were not processed together with ritonavir during the production of the sLBF because of the high glass transition temperatures of the polymers. The required processing temperatures would have led to degradation of the API.[43, 44] It may be possible that, for the precipitation inhibiting effect of PVP-VA and HPMC-AS, a fine dispersion of the polymer in the formulation is necessary from the beginning. Further investigation is needed to illuminate the mechanism behind this observation.

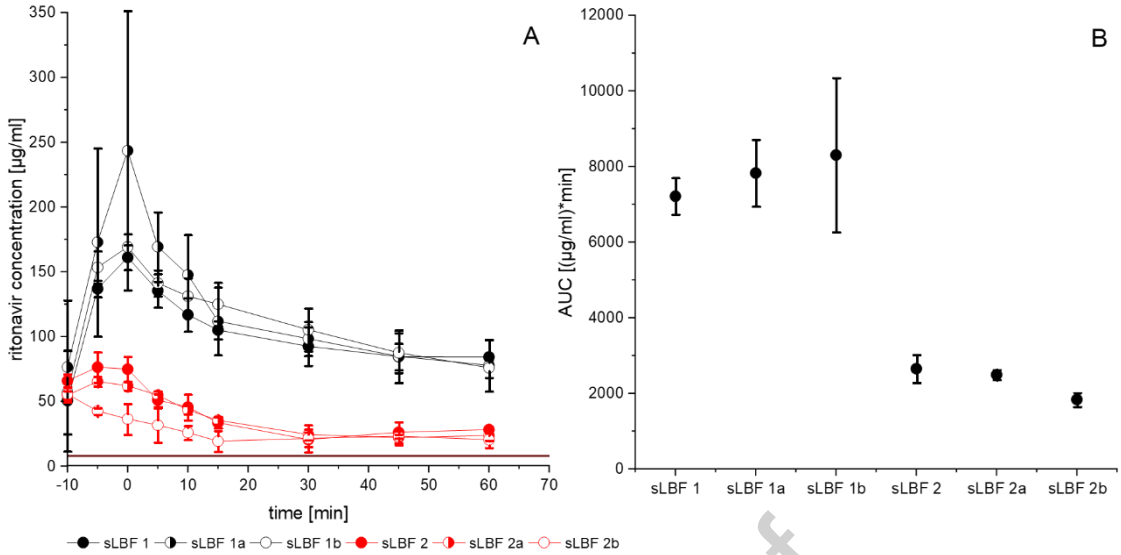

Figure 5. A: Ritonavir concentration over time during lipolysis. The red line marks the solubility of ritonavir in FaSSIF medium obtained from previous studies.[27] B: calculated AUC values of ritonavir concentration curves presented as mean ± SD (n=3).

3.4 *In vitro* lipolylis-permeation studies

Increasing the permeation rate of poorly soluble drugs is a major motivation for implementing LBFs and, thus, *in vitro* permeation studies using the ENA device were conducted.[22, 38] Interactions between the polymeric PIs and the artificial permeation membrane were observed, which resulted in the loss of membrane integrity. As the addition of PIs did not provide a positive effect on the stabilization of the supersaturated solution obtained in the lipolysis studies, experiments were only carried out for sLBFs 1 and 2 and pure ritonavir. Figures 6A and 6B present the concentration of ritonavir over time in the donor and receiver compartment. As expected, the solubility of ritonavir was significantly increased in the donor compartment for both sLBFs compared to the pure drug, confirming their solubilizing effect. By comparing the AUCs of the concentration in the donor compartment (Figure 6C), the extent of solubility enhancement for both formulations is apparent. The AUC of sLBF 1 is 13.1 times higher and that of sLBF 2 is 5.6 times higher than that of only ritonavir. When comparing the two formulations, sLBF 1 shows a 2.3 times higher AUC than sLBF 2 confirming the observations from the lipolysis studies that a higher amount of surfactant increases solubility without increasing the precipitation rate. For sLBF 1, the concentration of ritonavir decreases for 120 minutes and then increases again. This effect may be explained by the results of the digestion studies. Since the surfactant is digested first, the solubility of ritonavir is reduced quickly in the beginning. After 120 min, the digestion of triglycerides increases. The digested triglycerides may act as an emulsifying system for the API,[10] which can lead to an increase in the ritonavir solubility. The same trend can be observed for sLBF 2 between 15 and 30 min, which also fits the biphasic digestion curve of this formulation. Another potential explanation could be

resolubilization of amorphously precipitated ritonavir, as reported in literature[45]. Since it was not tested if the precipitate was amorphous, additional experiments are necessary to investigate these hypotheses.

Even though the differences in solubilization in the donor compartment are pronounced, the differences in permeation are lower. Both sLBF 2 and pure ritonavir show a lag time (15 and 30 min, respectively). It is hypothesized that the different lag times are due to the different initial concentration of ritonavir in the donor compartment. The permeation rates calculated from the data between 30 and 180 min show an increase by 71.1 % for sLBF 1 and by 29.8 % for sLBF 2 compared to pure ritonavir. Despite the significantly higher concentration in the donor medium, only the permeation rate of sLBF 1 is statistically significantly increased. While a correlation between increased permeation with higher dissolved drug concentration in the donor compartment seems to be obvious, the result also show that differences in the donor compartment do not automatically lead to comparable differences in the receiver compartment. Additionally, it should be noted that ritonavir shows pH dependent solubility,[46] which may influence the permeation behaviour of ritonavir. Indeed earlier lipolysis studies demonstrated that this is also the case during lipolysis experiments.[27]. Additional experiments using only surfactant as matrix for ritonavir should provide additional valuable information about sLBF permeation behaviour during digestion.

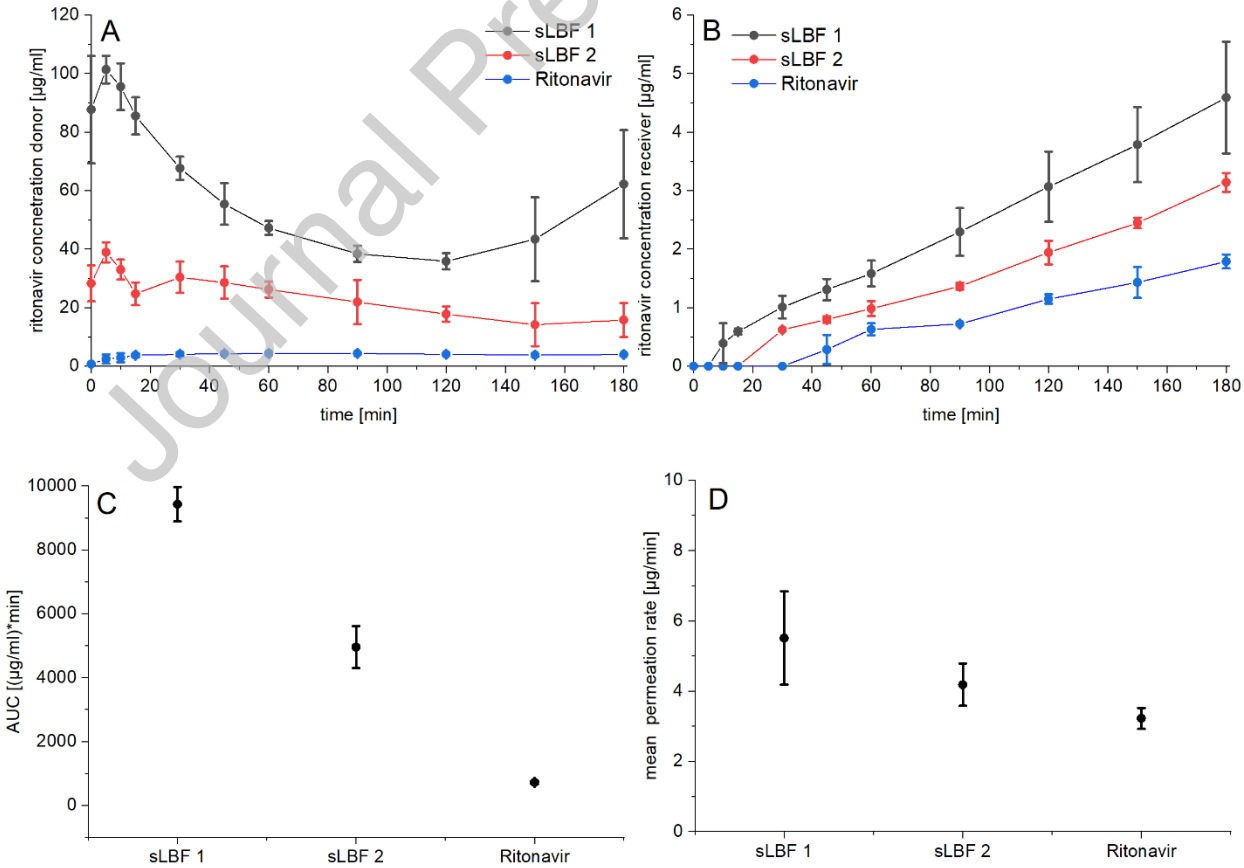

Figure 6. Ritonavir concentration curve in the donor medium (A) and receiver medium (B). Total AUC of the donor curves for all formulations tested (C) and mean permeation rate calculated from minute 30 to 180 to avoid lag time influence (D). All data presented as mean ± SD (n=3).

4. Conclusions

In this study, the lipolysis and permeation characteristics of sLBFs containing ritonavir and the influence of triglyceride and surfactant concentrations on these processes were investigated. The presence of common precipitation inhibitors, HPMC-AS and PVP VA, was also evaluated. Both investigated formulations showed increased permeation rates compared to pure ritonavir. Increasing the amount of a medium chain solid lipid component and decreasing the surfactant amount did not show any benefit in the lipolysis studies. Compared to a previous study,[27] where long-chain mono- and triglycerides were combined to increase the ritonavir concentration during lipolysis, the results from this study indicate that higher surfactant concentrations, e.g., in a Type IV formulation, may also lead to higher permeation rates for ritonavir and potentially other APIs. Further investigations including other APIs are necessary to better understand these systems. Surprisingly, higher ritonavir concentrations during digestion in the donor compartment did not automatically lead to significantly higher permeation rates even though flux and AUC were strongly increased. This should be taken into account by formulation developers when considering if the risks of increased surfactant concentrations (intestinal intolerance) are worth of the limited permeation increase.

Interestingly, the precipitation inhibitors did not show any significant effects. This should be further investigated in order to better understand the mechanism of action of these systems. As mentioned before, adding the polymers during the manufacturing of sLBFs could lead to stronger interactions of ritonavir with the polymers and impact precipitation. Unfortunately, this was not possible within this study as the processing temperatures of the polymers would lead to degradation of ritonavir.[43, 44] The use of polymers with lower glass transition temperatures or plasticizers may be an option to overcome this problem. Another possible solution to incorporate the polymers at reduced temperatures into the sLBF would be the introduction of mechanical forces, e.g., *via* twin screw extrusion. Following this process, the sLBFs could further be processed directly *via* tableting or 3D printing. Further studies to investigate whether *in vitro-in vivo* correlations can be derived for sLBFs using these experimental set-ups are essential.

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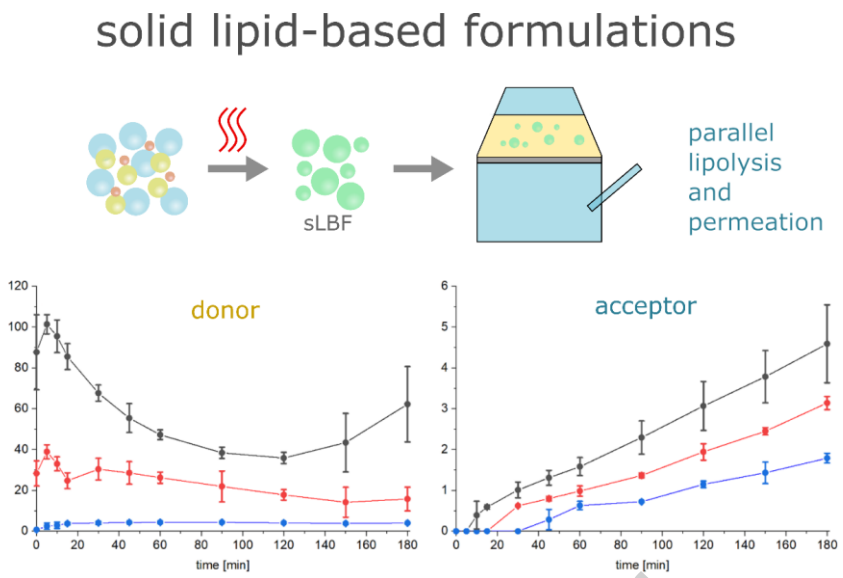
Graphical Abstract



Declaration of interest: Ioannis I. Andreadis is currently an employee of AstraZeneca PLC. Christel A. S. Bergström is founder of the company Enphasys AB, which holds the patent for the ENA device.