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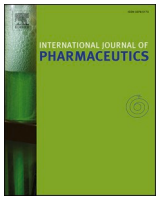

Self-emulsifying drug delivery systems (SEDDS): *In vivo*-proof of concept for oral delivery of insulin glargine

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ABSTRACT

In spite of recent progress made in the field of peptide and protein delivery, oral administration of insulin and similar drugs remains a challenge. In this study, lipophilicity of insulin glargine (IG) was successfully increased via hydrophobic ion pairing (HIP) with sodium octadecyl sulfate to enable incorporation into self-emulsifying drug delivery systems (SEDDS). Two SEDDS formulations (F1: 20% Labrasol® ALF, 30% polysorbate 80, 10% Croduret 50, 20% oleyl alcohol, 20% Maisine® CC; F2: 30% Labrasol® ALF, 20% polysorbate 80, 30% Kolliphor® HS 15, 20% Plurol® oleique CC 497) were developed and loaded with the IG-HIP complex. Further experiments confirmed increased lipophilicity of the complex, achieving $LogD_{SEDDS/release\ medium}$ values of 2.5 (F1) and 2.4 (F2) and ensuring sufficient amounts of IG within the droplets after dilution. Toxicological assays indicated minor toxicity and no toxicity inherent to the incorporated IG-HIP complex. SEDDS formulations F1 and F2 were administered to rats via oral gavage and resulted in a bioavailability of 0.55% and 0.44%, corresponding to a 7.7-fold and 6.2-fold increased bioavailability, respectively. Thus, incorporation of complexed insulin glargine into SEDDS formulations provides a promising approach to facilitate its oral absorption.

1. Introduction

Diabetes mellitus is a group of metabolic diseases that are characterized by high blood glucose levels. If left untreated, it can lead to serious long-term consequences such as kidney dysfunction, nerve damage, cardiovascular disease and even death. The complications can be reduced by ensuring normal blood glucose levels, mostly achieved through the administration of insulin, an endocrine peptide hormone, which regulates glucose uptake into cells and reduces blood glucose via inhibition of gluconeogenesis and lipolysis (Wilcox, 2005). Insulin, like many other therapeutic peptides, has to be administered subcutaneously due to insufficient oral bioavailability, which can be risky and is often associated with pain. In fact, about 60% of patients fail to control their blood glucose levels over a long period of time, partly due to complicated dosing as well as difficult administration (Saaddine et al., 2006). In order to address this shortcoming, the pharmaceutical industry has been working on 'injectable-to-oral-conversions' for decades. In the 2000s Nobex developed a hexyl-insulin monoconjugate (HIM2) for oral delivery followed by Emisphere Technologies utilising their permeation enhancer SNAC for oral insulin delivery and more recently Oramed Pharmaceuticals developed insulin capsules with a permeation enhancer and enzyme inhibitors. All these attempts, however, failed so far in clinical trials as the oral bioavailability was too low.

Most therapeutic peptides exhibit low gastrointestinal membrane permeability and are prone to enzymatic degradation, hampering their oral administration (Pereira de Sousa and Bernkop-Schnürch, 2014). The use of lipid-based nanocarriers (LBN), such as self-emulsifying drug delivery systems (SEDDS), presents a path to oral peptide delivery,

Abbreviations: MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); AAC, area above the curve; AUC, area under the curve; BA, bioavailability; DLS, dynamic light scattering; HIP, hydrophobic ion pair; HPLC, high performance liquid chromatography; IG, insulin glargine; IU, international unit; LBN, lipid-based nanocarriers; PA, pharmacological activity; PDI, polydispersity index; PE, Precipitation efficiency; SEDDS, self-emulsifying drug delivery systems; SDS, sodium dodecane sulfonate; SIF, simulated intestinal fluid; SOS, sodium octadecyl sulfate; TFA, trifluoroacetic acid.

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providing improved permeation and protection against enzymatic degradation (Griesser et al., 2018; Hetényi et al., 2017). However, most biopharmaceuticals are too hydrophilic to be incorporated into LBN. Hydrophobic ion pair (HIP) formation can be used to increase the lipophilicity of these drugs and to facilitate their incorporation into SEDDS. HIPs are formed by combining the API with an appropriate counter ion exhibiting hydrophobic substructures, thus leading to precipitation in aqueous media due to the water insolubility of the uncharged complex (Ristroph and Prud'homme, 2019). Preceding studies have demonstrated that this technique can also be applied to insulin and results of first in vivo studies are encouraging (Griesser et al., 2017; Hetényi et al., 2017; Karamanidou et al., 2015; Nazir et al., 2019; Noh et al., 2023). As insulin exhibits just six cationic moieties, however, the number of anionic hydrophobic counterions that can be attached to this drug to raise its lipophilicity is limited. In contrast to native insulin, insulin glargine (IG) offers two additional cationic substructures for HIP formation. Produced from genetically modified microorganisms, IG is used as a long-acting insulin for type I and type II diabetes. It differs from human insulin by replacement of asparagine with glycine in position 21 of the A-chain and addition of two easily chargeable arginine residues ($pK_a \sim 14$) likely increasing the potential for HIP in comparison to unmodified insulin (Fitch et al., 2015). In order to evaluate the potential of this analogue for the design of advanced SEDDS, it was the objective of this study to ion pair IG serving as model for peptide drugs with various hydrophobic counterions aiming for a substantial increase in lipophilicity. Subsequently, the most promising HIP was incorporated into SEDDS and following toxicological investigations, SEDDS were administered to Sprague Dawley rats via oral gavage to determine their in vivo efficacy.

2. Materials and methods

2.1. Materials

IG (Lantus®) was purchased from Sanofi, dialyzed at pH 4 (molecular weight cut-off 1 kDa) and subsequently lyophilized. Labrasol® ALF (caprylocaproyl polyoxyl-8-glycerides), Maisine® CC (glyceryl monolinoleate), Peceol™ (glyceryl monooleate, type 40), Plurol® oleique CC 497 (polyglyceryl-3 dioleate), Labrafac™ lipophile WL 1349 (medium-chain triglycerides), Transcutol® HP (diethylene glycol monoethyl ether) and Labrafil® M 2125 CS (lineleoyl polyoxyl-6 glycerides) were kindly provided by Gattefossé (Saint-Priest, France). Polysorbate 80 was purchased from Merck KGaA (Darmstadt, Germany). Jordapon® (sodium cocoyl isethionate) and Kolliphor® HS 15 (PEG-15 hydroxystearate) were received from BASF (Ludwigshafen, Germany). Croduret™ 50 (PEG-40 hydrogenated castor oil) was kindly provided by Croda (Nettetal, Germany). Hydrochloric acid (32%, w/w) was obtained from Carl Roth (Karlsruhe, Germany). Red blood cell concentrate was received from Tirol Kliniken GmbH (Innsbruck, Austria). Trifluoroacetic acid, sodium octadecyl sulfate and sodium taurocholate were purchased from Thermo Scientific (Vienna, Austria). All solvents (HPLC grade), oleyl alcohol and all other chemicals were obtained from Sigma-Aldrich (Vienna, Austria).

2.2. Methods

2.2.1. HPLC quantification of IG

A Hitachi Chromaster HPLC-system consisting of a 5160 pump, 5260 autosampler, 5310 column oven and 5430 diode array detector (VWR Hitachi, Vienna, Austria) was used to quantify IG. A LiChrospher® 100 RP-18 column (125 × 4.6 mm, 5 µm) was utilized as the stationary phase in combination with a LiChroCart 4–4 HPLC guard column. The mobile phase consisted of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). An elution gradient was used starting at 70% A until 5.5 min, then set to 60:40 (A:B) at 6 min, before re-equilibrating the system back to 70:30 (A:B) at 11.5 min for a total of 16 min at a flow rate of 1 ml/min. Until injection, samples were stored at 10 °C within the auto sampler. The injection volume was 20 µl, column temperature was set to 40 °C and detection wavelength was 214 nm with an elution time of 9.8 min. The HPLC method resulted in an adequate peak shape and calibration curves exhibited sufficient linearity over a range from 0.008 to 0.5 mg/ml ($R^2 = 0.999$).

2.2.2. Hydrophobic ion pairing

In order to increase lipophilicity of IG, hydrophobic ion pairs were formed. Thereby, IG was dissolved in 0.01 M HCl in a concentration of 1 mg/ml to obtain a completely ionized IG with 8 positive charges. Applied surfactants (Table 1) were dissolved in water in concentrations corresponding to charge ratios of 1:0.5, 1:1 and 1:2 (IG:surfactant) after combination of equal volumes of IG and surfactant solution. Hereby, a charge ratio of 1:1 translates to a ratio of 1 mole of IG to 8 mole of surfactant. Samples were shaken at 400 rpm for 2 h at 25 °C utilizing a Thermomixer (Eppendorf, Austria). Precipitated complexes were separated from the supernatant by centrifugation at 12,045 g for 10 min with a Minispin (Eppendorf, Austria). Precipitation efficiency (PE) was calculated using equation (1):

$$PE\ \left[\%\right] = 100 \times \left(1 - \frac{c_{IG\ after\ HIP}}{c_{IG\ before\ HIP}}\right) \quad (1)$$

The obtained pellets were washed with water, subsequently lyophilized (Labconco FreeZone 6 Liter Benchtop Freeze Dry System) and stored in the freezer at −20 °C until further use.

2.2.3. Determination of log $D_{n\text{-}octanol/water}$

For the determination of the log $D_{n\text{-}octanol/water}$, HIP complexes were dissolved in 400 µl of a 1:1 (v/v) mixture of n-octanol/water and incubated at 37 °C while shaking at 500 rpm for 24 h. After centrifugation of the samples for 10 min at 12,045 g, aliquots of each phase were withdrawn and analyzed via HPLC. The log $D_{n\text{-}octanol/water}$ was calculated based on equation (2):

$$log\ D_{n-octanol/water} = log \frac{c_{n-octanol}}{c_{water}} \quad (2)$$

2.2.4. Solubility studies

To identify SEDDS components suitable for dissolution of HIP complexes, different organic solvents and surfactants commonly used as SEDDS excipients were tested (Labrafac™ lipophile WL 1349, Labrafil® M 2125 CS, Labrasol® ALF, Maisine® CC, oleyl alcohol, Plurol® oleique CC 497, propylene glycol, Transcutol® HP, polysorbate 80). In brief, excipients were added to a surplus of complex and treated with ultrasound for 10 min. Samples were then centrifuged for 10 min at 12,045 g and the supernatant was analyzed via HPLC.

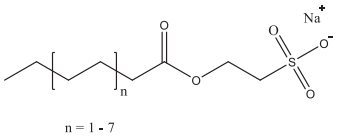
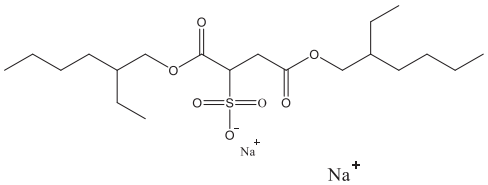
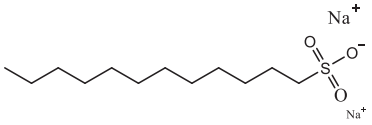
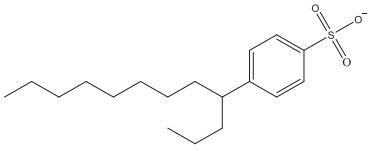
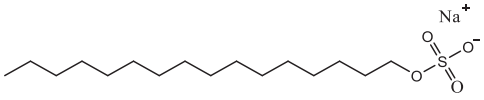
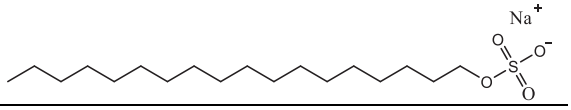
2.2.5. Preparation and characterization of SEDDS

Based on the results of the dissolution studies, SEDDS preconcentrates were prepared accordingly. Thereby, solvents, co-solvents and surfactants were mixed and visually investigated regarding homogeneity. Final formulations were diluted 1:50 (v/v) with simulated intestinal fluid (SIF, 50 mM KH_2PO_4) pH 6.8 and further characterized regarding droplet size, polydispersity index (PDI) and zeta potential (NanoBrook 90 Plus PALS; Brookhaven) immediately after emulsification and after incubation for 4 h at 37 °C while shaking at 300 rpm. This characterization was performed with and without incorporation of the HIP complex into the SEDDS preconcentrate, as described below.

2.2.6. Payload and log $D_{SEDDS/release\ medium}$

To confirm sufficient solubility of the complex in SEDDS formulations, the highest achievable payload was determined via HPLC. Thereby, SEDDS containing an excess of HIP were treated with ultrasound for 2 h at 37 °C before incubating samples overnight at 500 rpm and 37 °C. Samples were then centrifuged at 12,045 g for 15 min and the

Table 1
Surfactants used for hydrophobic ion pairing.

Surfactant	Chemical structure
Sodium cocoyl isethionate	
Sodium docusate	
Sodium dodecane sulfonate	
Sodium dodecylbenzene sulfonate	
Sodium hexadecyl sulfate	
Sodium octadecyl sulfate	

supernatant was analyzed for dissolved IG. Furthermore, log $D_{SEDDS/release\ medium}$ was determined by measuring the solubility of the complex in SIF pH 6.8 as described above and calculated using equation (3):

$$log\ D_{SEDDS/release\ medium} = log \frac{c_{SEDDS}}{c_{SIF\ pH\ 6.8}} \quad (3)$$

2.2.7. Hemolytic activity studies

To evaluate the toxicity of unloaded and loaded SEDDS on cell membranes, an in vitro hemolysis assay was used on human red blood cells. In brief, red blood cell concentrate (Tirol Kliniken GmbH, Innsbruck, Austria) was diluted 1:200 (v/v) with sterile glucose-HEPES buffer pH 7.4. SEDDS were then diluted with buffer resulting in concentrations of 0.0005, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1 and 0.25% (v/v). Thereafter, 0.5 ml of SEDDS solution were mixed with the same volume of diluted erythrocyte concentrate and incubated for 4 h at 37 °C while shaking at 150 rpm. Hereby, 0.5 ml of a 0.5% (w/v) Triton X-100 solution and 0.5 ml of buffer, both mixed with 0.5 ml of diluted erythrocyte concentrate, served as positive and negative control, respectively. After samples were centrifuged at 500 g for 10 min, 100 µl of the supernatant were transferred into a 96-well plate and sample absorbance was measured at a wavelength of 415 nm using a microplate reader (Tecan Spark).

The extent of hemolysis as percentage (% H) was determined by using equation (4):

$$\%\ H = \frac{Abs_{Test} - Abs_{Neg}}{Abs_{Pos} - Abs_{Neg}} \times 100 \quad (4)$$

where Abs_{Test} is absorbance of the test sample, Abs_{Neg} is absorbance of the negative control and Abs_{Pos} is absorbance of the positive control.

2.2.8. Cell viability studies

Potential cytotoxic effects of 0.01, 0.05, 0.1 and 0.25% (v/v) loaded and unloaded SEDDS were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Kumar et al., 2018). In brief, a 96-well plate was seeded in a density of 5×10^4 Caco-2 cells per well. Thereafter, cells were incubated for 5 days in minimal essential media (MEM) supplemented with 10% (v/v) heat inactivated fetal calf serum and penicillin/streptomycin solution (100 units/0.1 mg/l) at 37 °C and 95% humidity in an atmosphere of 5% CO_2 to build a monolayer. After removal of MEM, cells were washed twice with 100 µl of sterile glucose-HEPES buffer pH 7.4. Then, 100 µl of SEDDS samples were added to each well and incubated for 24 h at 37 °C. Wells were washed with 100 µl of sterile glucose-HEPES buffer following the removal of samples. After addition of 100 µl of MTT solution (0.5 mg/ml MTT in sterile glucose-HEPES buffer), plates were incubated light-protected at 37 °C for 2 h. MTT solution was carefully removed and 120 µl of DMSO was added to dissolve formazan crystals. After placing it in the orbital shaker incubator at 150 rpm for 10 min, samples were analyzed by transferring 100 µl into a 96-well plate and measuring the absorbance at 570 nm with a multiplate reader (Tecan Spark). Glucose-HEPES buffer and 0.1% Triton-X solution served as positive and negative controls, respectively.

Cell viability was determined by using equation (5):

$$Cell\ viability\ [\%] = \frac{Abs_{Test} - Abs_{Pos}}{Abs_{Neg} - Abs_{Pos}} \times 100 \quad (5)$$

where Abs_{Test} is absorbance of the test sample, Abs_{Neg} refers to the absorbance of the negative control and Abs_{Pos} is absorbance of the positive control.

2.2.9. In vivo study

The procedure was approved by the Animal Ethical Committee of Vienna, Austria and adhered to the Principles of Laboratory Animal Care. Male Sprague Dawley rats with a mean body weight of 250 – 350 g were obtained from Janvier Labs (Le Genest-Saint-Isle, France). The rats were randomly divided into 4 groups (n = 3) and fasted for 12 h within

metabolic cages prior to the experiment having free access to water at any time. The first group received a dose of 0.5 IU/kg bodyweight of sterile filtered IG solution via intravenous injection. A solution of IG in a dose of 85 IU/kg bodyweight was given to the second group via oral gavage. SEDDS formulations F1 and F2 containing the HIP in a dose of 85 IU/kg bodyweight were administered via oral gavage to the third and fourth group, respectively. Hereby, SEDDS preconcentrates were emulsified in a ratio of 1:5 (SEDDS:phosphate buffered saline pH 6.8, v/v) before application, as described by Rao et al. (Rao et al., 2008). During the experiment, rats had free access to water. At predetermined timepoints, blood samples were taken from the tail vein and spiked with 5 µl of a 3.6% (w/v) EDTA solution to prevent blood clotting. Thereafter, samples were centrifuged at 1,677 g for 15 min and plasma was separated for quantification of IG via ELISA (IV2-101E, Invitron, Monmouth, UK) according to the manufacturer's protocol. In addition, blood glucose level was measured using both Contour next (Contour, Vienna, Austria) and OneTouch Verio (Onetouch, Vienna, Austria). Due to high similarity of obtained data for both devices, the mean value of measurements was taken into account.

Bioavailability (BA) was calculated based on IG plasma concentration by using equation (6):

$$BA\ [\%] = \frac{AUC_{sample} \times dose_{i.v.}}{AUC_{i.v.} \times dose_{sample}} \times 100 \quad (6)$$

where AUC_{sample} is the area under the curve of test sample, $AUC_{i.v.}$ is the area under the curve of i.v. sample, $dose_{i.v.}$ is the dose given i.v. and $dose_{sample}$ is the dose of test sample.

In addition, pharmacological availability was calculated according to Goo et al. (Goo et al., 2022). Each blood glucose level was calculated relative to the initial blood glucose level at 0 h. The pharmacological availability (PA) was calculated by using equation (7):

$$PA\ [\%] = \frac{AAC_{sample} \times dose_{i.v.}}{AAC_{i.v.} \times dose_{sample}} \times 100 \quad (7)$$

where AAC_{sample} is the area above the curve of test sample, $AAC_{i.v.}$ is the area above the curve of i.v. sample, $dose_{i.v.}$ is the dose given i.v. and $dose_{sample}$ is the dose of test sample.

2.2.10. Statistical analysis

Statistical data analyses were applied using Student's *t*-test between two independent means. Hereby, p-values < 0.05 were considered significant and $p < 0.01$ as very significant. Results are means of at least three experiments ± standard deviation (SD).

3. Results and discussion

3.1. Hydrophobic ion pairing and log $D_{n\text{-}octanol/water}$

For the application of hydrophobic ion pairing, oppositely charged groups are needed between the API and counterion. IG exhibits eight basic moieties compared to six for regular insulin, due to the addition of two easily chargeable arginine residues (Fitch et al., 2015). These functional groups are ionized at pH 2 and can be used to form water-insoluble complexes with negatively charged groups of various surfactants (Zupančič and Bernkop-Schnürch, 2017). In this study, the potential of different commercially available surfactants (Table 1) to increase the lipophilicity was assessed based on their ability to precipitate IG. Results are shown in Fig. 1, demonstrating the highest precipitation efficiencies of > 98% for sodium cocoyl isethionate (SCI) in a charge ratio of 1:2 (IG:counterion), sodium docusate (1:1), sodium dodecane sulfonate (SDS, 1:1 and 1:2) and sodium octadecyl sulfate (SOS, 1:1). Sodium dodecylbenzene sulfonate (1:1) and sodium hexadecyl sulfate (1:1) also resulted in high precipitation efficiencies of 90% and 84%, respectively. With the exception of SDS and SCI, an excess of surfactant (charge ratio 1:2) resulted in a decrease of precipitation efficiency, likely caused by dissolution of the complex via micelle formation (Choi and Park, 2000; Griesser et al., 2017; Wibel et al., 2020). Addition of sodium dodecane sulfonate in a charge ratio of 1:2 did not result in a lower precipitation efficiency compared to sodium hexadecyl sulfate and SOS. SCI resulted in its peak value at a charge ratio of 1:2, possibly related to steric hindrance of the surfactant molecule. When surfactants were added in a ratio of 1:0.5, a sharp decrease to below 60% was observed in all cases, independent of surfactant structure. In general, all surfactants yielded sufficient precipitation efficiencies and were therefore further characterized.

To evaluate the increase in lipophilicity of IG via HIP, the log $D_{n\text{-}octanol/water}$ was determined. Log D is a key parameter to predict the behavior of the HIP complex after subsequent incorporation into SEDDS, as it provides an estimate about the solubility in lipophilic excipients and its stability (Dumont et al., 2020). SOS showed the highest increase in log $D_{n\text{-}octanol/water}$ in a charge ratio of 1:1 (IG:counterion) from −1.5 (native IG) to 1.9, followed by sodium hexadecyl sulfate with 1.8 (1:1) (Fig. 2). Among different charge ratios of surfactants, no significant difference was observed for most of them. Both sodium hexadecyl sulfate and SOS resulted in lower log $D_{n\text{-}octanol/water}$ values, possibly as a result of insufficient complex formation (charge ratio of 1:0.5) and residual surfactants (charge ratio of 1:2) despite a washing step before lyophilization. The addition of sodium docusate to IG in a ratio of 1:2

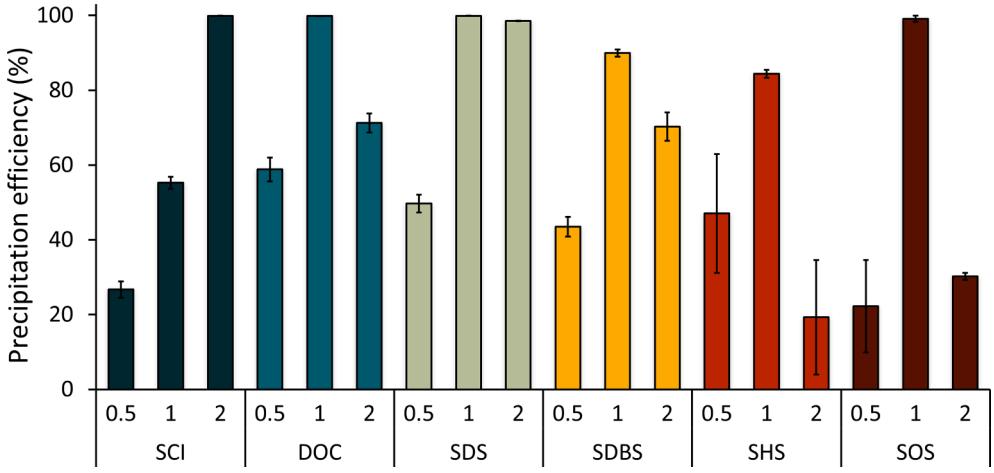


Fig. 1. Precipitation efficiency (%) of IG ion paired with various counterions at different charge ratios (1:X; IG:counterion). Indicated values are means (n = 3) ± SD. SCI: sodium cocoyl isethionate, DOC: sodium docusate, SDS: sodium dodecane sulfonate, SDBS: sodium dodecylbenzene sulfonate, SHS: sodium hexadecyl sulfate, SOS: sodium octadecyl sulfate.

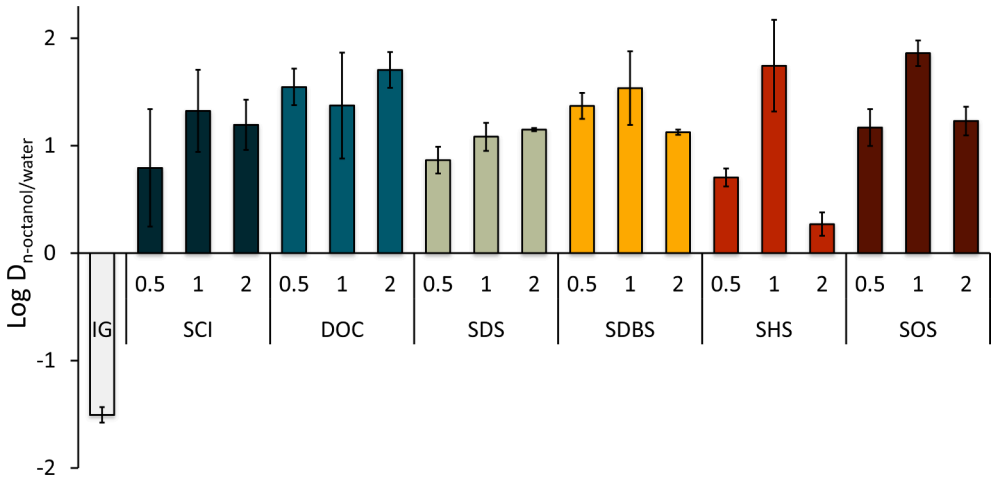


Fig. 2. Log $D_{n\text{-octanol/water}}$ of IG ion paired with various counterions at different charge ratios (1:X; IG:counterion). Indicated values are means (n = 3) ± SD. IG: insulin glargine, SCI: sodium cocoyl isethionate, DOC: sodium docusate, SDS: sodium dodecane sulfonate, SDBS: sodium dodecylbenzene sulfonate, SHS: sodium hexadecyl sulfate, SOS: sodium octadecyl sulfate.

resulted in a log $D_{n\text{-octanol/Water}}$ of 1.7, consistent with previous studies utilizing regular insulin (Griesser et al., 2017). Thus, no difference between regular insulin and IG was observed regarding log D utilizing the same counterion. Consequently, further studies were conducted with IG:SOS complex in a charge ratio of 1:1, exhibiting the highest increase in lipophilicity.

3.2. SEDDS development

For the development of SEDDS formulations, various solvents, co-solvents and surfactants were investigated regarding their solubility properties of complexed IG. The results of preliminary solubility studies are presented in Fig. 3. SEDDS were designed with regard to critical parameters such as a sufficient solubility of the HIP complex, suitable droplet size, polydispersity index and stability after emulsification. Within this study, the focus was set on very lipophilic formulations, which are larger by nature and therefore exceed the usually targeted droplet size of 200 nm (Griesser et al., 2018). Hence, formulations did not contain organic solvents that are rapidly released from SEDDS in vivo (Jörgensen et al., 2020). Both unloaded formulations exhibited a stable droplet size of around 200 – 300 nm over 4 h and were considered monodisperse with PDI values below 0.2. In SEDDS formulation F1, Maisine® CC was used as oily vehicle, comprising a mixture of mono-, di- and triglycerides of linoleic and oleic acids. Long-chain triglycerides have been reported to facilitate uptake via the lymphatic transport system and therefore increase bioavailability and absorption (Bandyopadhyay et al., 2012; Holm et al., 2006, 2003; Khoo et al., 1998). Plurol® oleique CC 497, which exhibits a comparable long chain fatty acid backbone, was added to F2 for a similar reason. In both developed formulations, Labrasol® ALF was incorporated as permeation enhancer to increase uptake into cells, previously having shown the ability to improve the intestinal absorption of insulin (Eaimtrakarn et al., 2002; McCartney et al., 2019; Sha et al., 2005). Polysorbate 80, Croduret™ 50, Kolliphor® HS 15 and oleyl alcohol were used as lipophilic co-solvents and surfactants to ensure rapid emulsification and subsequent stability. Although the HIP was poorly soluble in polysorbate 80 and oleyl alcohol, these excipients were nonetheless chosen for formulation F1 since they were responsible for sufficiently high emulsifying properties of this formulation. The detailed composition of SEDDS formulations can be found in Table 2.

Before and after incorporation of the IG:SOS complex, SEDDS were emulsified with SIF pH 6.8 and analyzed after 0 h and 4 h. As displayed in Table 3, F2 increased in droplet size after addition of the complex, possibly caused by release of surfactant molecules. In contrast, F1

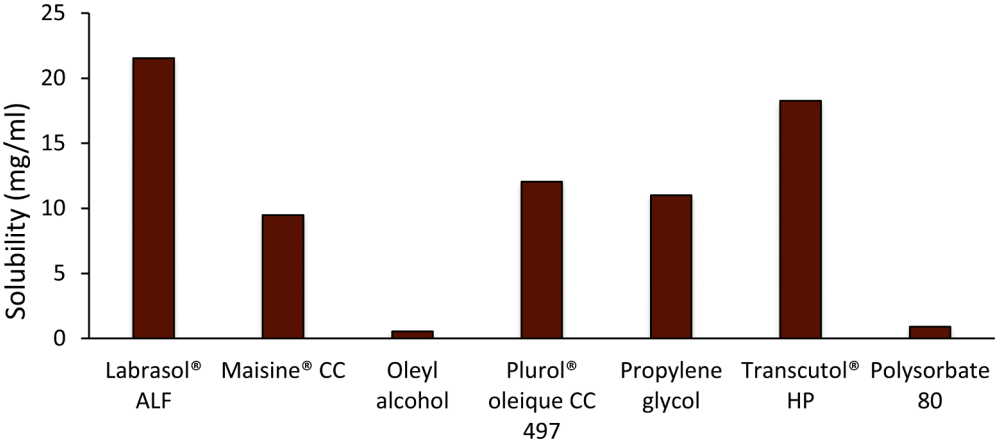


Fig. 3. Solubility studies of IG ion paired with SOS in a charge ratio of 1:1 in various surfactants determined via HPLC. HIP complex was not soluble in Labrafac™ lipophile WL 1349 and Labrafil® M 2125 CS. IG: insulin glargine.

Table 2
Composition of SEDDS formulations (%. v/v).

F.	Labrasol® ALF	Polysorbate 80	Croduret™ 50	Kolliphor® HS 15	Oleyl alcohol	Plurol® oleique CC 497	Maisine® CC
F1	20	30	10		20		20
F2	30	20		30		20	

Table 3
Droplet size (DS), polydispersity index (PDI) and zeta potential (ZP) of empty and HIP-loaded SEDDS formulations after emulsification with SIF pH 6.8 in a ratio of 1:50 (v/v). Indicated values are means (n = 3) ± SD.

Form.	SEDDS (unloaded)		SEDDS (HIP-loaded)	
	0 h	4 h	0 h	4 h
		F1		
DS (nm)	254.0 ± 16.2	217.1 ± 31.9	200.5 ± 16.9	193.0 ± 10.1
PDI	0.12 ± 0.12	0.07 ± 0.08	0.22 ± 0.02	0.17 ± 0.11
ZP (mV)	−1.39 ± 2.18	−2.20 ± 0.55	−2.54 ± 0.60	−0.78 ± 1.33
		F2		
DS (nm)	306.1 ± 29.2	301.8 ± 6.4	347.7 ± 7.7	326.6 ± 14.2
PDI	0.12 ± 0.06	0.10 ± 0.06	0.11 ± 0.11	0.16 ± 0.07
ZP (mV)	−1.72 ± 1.37	−0.61 ± 2.14	−3.20 ± 0.99	−2.62 ± 0.63

decreased slightly in size. This may be due to the fact that drug loading can contribute to droplet stabilization, facilitating a smaller droplet size, as observed in preceding studies (Vasconcelos et al., 2018). Both formulations remained stable over 4 h, independent of complex incorporation, underlining their structural integrity after emulsification. HIP loading showed no influence on zeta potential and revealed a near to neutral surface charge of investigated formulations.

Formulations were further evaluated regarding their maximum solubility and log $D_{SEDDS/release\ medium}$. F1 and F2 exhibited a solubility of 7.7 mg/ml and 6.4 mg/ml, respectively, comparable to other recent studies (Fig. 4) (Noh et al., 2023). This underlines the advantages of increased lipophilicity of the HIP, considering that preceding research mostly relied on more hydrophilic SEDDS formulations without a lipophilic core, which can lead to premature release and thus precipitation of the API (Jörgensen et al., 2020; Karamanidou et al., 2015). In addition, the release may be reduced by the high initial lipophilicity of the complex (Dumont et al., 2020). The formulations showed log $D_{SEDDS/release\ medium}$ values of 2.5 (F1) and 2.4 (F2), superior to comparable literature values within the same release medium (Noh et al., 2023). Log D values above 2 indicate that most of the HIP complex remains within the oily phase during the gastrointestinal passage facilitating protection from enzymatic degradation (Hetényi et al., 2017).

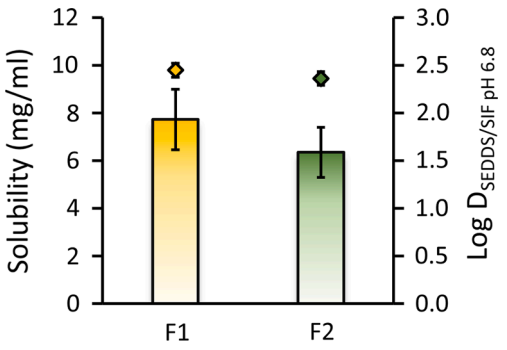


Fig. 4. Maximum solubility determination (bars, mg/ml) and Log $D_{SEDDS/SIF\ pH\ 6.8}$ (◆) of IG ion paired with SOS in a charge ratio of 1:1 (IG:SOS) in formulations F1 and F2. Indicated values are means (n = 3) ± SD.

3.3. Hemolytic activity and cell viability

The in vitro hemolysis assay is a reliable method to evaluate possible interactions between LBN and cell membranes (Lam et al., 2019). Hereby, erythrocytes serve as substitute for the lipid bilayer membrane surrounding endosomes, providing information about endosomal escape as well as cell toxicity (Evans et al., 2013). SEDDS naturally contain surfactants, that can compromise cell membrane integrity due to membrane solubilization (Ujhelyi et al., 2012). As shown in Fig. 5, F2 showed no hemolytic activity up to a concentration of 0.005%, whereas hemolytic activity of F1 increased concentration-dependent to 0.01%, achieving full hemolysis likely associated to the incorporation of oleyl alcohol. Full hemolysis was reached by F2 at a higher concentration of 0.1%, which might be due to the non-toxicity associated with polyglycerol surfactants (Shahzadi et al., 2021). In general, only minor differences in hemolytic activity between unloaded and loaded SEDDS could be observed, suggesting that the HIP complex itself is not toxic.

The cytotoxicity was further evaluated on Caco-2 cells utilizing MTT assay. Due to its intestinal origin, Caco-2 cell monolayers provide similar properties to enterocytes found in the intestine (Kauffman et al., 2013; Simon-Assmann et al., 2007). According to López-García et al., a cell viability above 80% is considered as non-cytotoxic, within 80–40% as weakly/moderately and below 40% as strongly cytotoxic (López-García et al., 2014). F2 demonstrated non-cytotoxicity in a concentration of 0.05% (v/v) after 24 h, whereas F1 resulted in a cell viability of just below 80% (Fig. 6). Increasing the SEDDS concentrations resulted in a lower cell viability in both formulations with no apparent impact of the incorporated complex. However, in vitro data may not correlate accurately to in vivo conditions, as the protective mucus layer of the intestinal epithelium is not addressed for in this experimental setup. In addition, SEDDS concentration decreases in vivo over time because of absorption and digestion processes.

3.4. In vivo study

In spite of recent progress made in the field of peptide and protein delivery, oral administration of insulin and similar drugs remains a major challenge. Currently there is no alternative to subcutaneous injections of insulin available for the treatment of diabetes mellitus (Fonte et al., 2013; Menzel et al., 2018). Although there have been proof of concept studies on oral administration, results have yet to meet expectations due to harsh pH environments, enzymatic degradation and glutathione induced conversion as well as limited permeability through the intestinal epithelium (Carino and Mathiowitz, 1999; Schmitz et al., 2006). In this context, the lipophilicity of the formulation is considered a key parameter for improved bioavailability. Therefore, the novel developed IG complexes were used to evaluate the pharmacological effect after administration via oral gavage. Pharmacokinetic data are summarized in Table 4, blood glucose levels are shown in Fig. 7 and IG plasma profiles are presented in Fig. 8.

The aqueous solution of native IG did not elicit a pharmacological effect in form of lowering blood glucose levels, easily explained by its rapid degradation under the harsh conditions found in the gastrointestinal tract (Leonaviciute and Bernkop-Schnürch, 2015). As complexation and subsequent incorporation into SEDDS provides protection against these barriers, a higher bioavailability could be assumed and was found depicted by 7.7- and 6.2-fold increases for F1 and F2, respectively (Hetényi et al., 2017). Hereby, F2 represented a more hydrophilic formulation possibly decreasing emulsification time and thereby

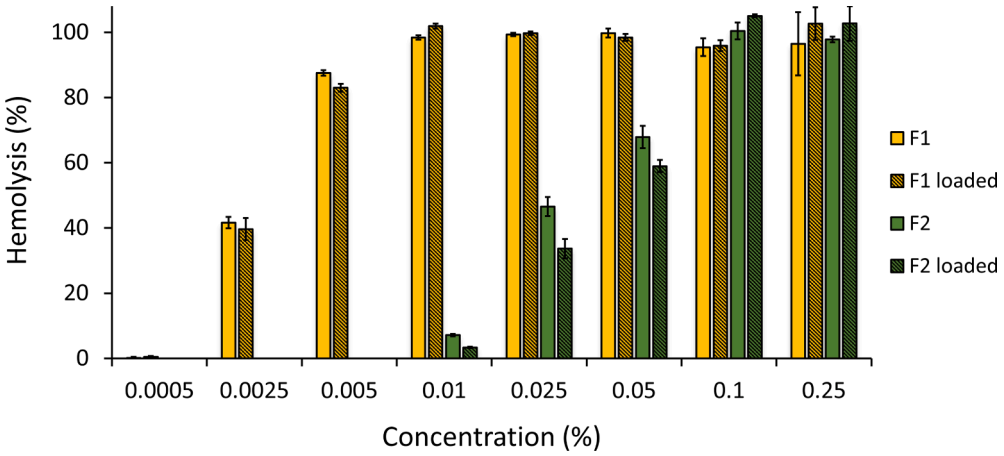


Fig. 5. Hemolysis (%) of fresh human erythrocytes after 4 h incubation at 37 °C with F1 (yellow bars), F1 loaded (yellow-striped bars), F2 (green bars) and F2 loaded (green-striped bars) in indicated concentrations. Triton X-100 (0.5%) served as positive control and glucose-HEPES buffer pH 7.4 as negative control. Indicated values are means (n ≥ 3) ± SD.

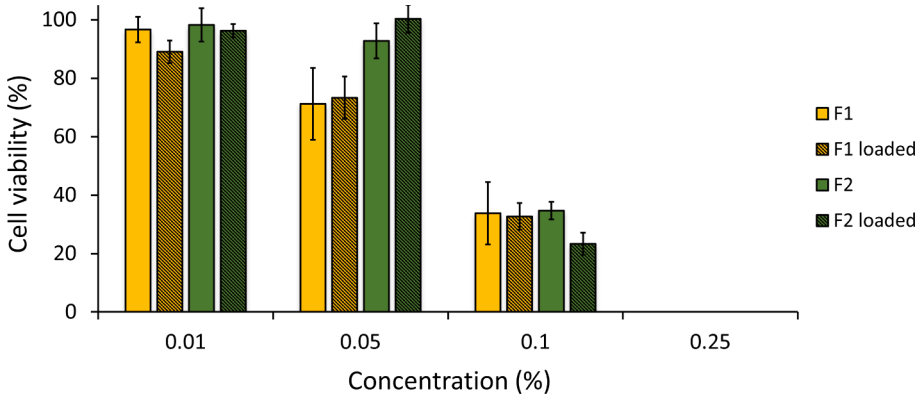


Fig. 6. Cell viability (%) of Caco-2 cells after 24 h incubation at 37 °C with F1 (yellow bars), F1 loaded (yellow-striped bars), F2 (green bars) and F2 loaded (green-striped bars) in indicated concentrations. Triton X-100 (0.1%) served as positive control and glucose-HEPES pH 7.4 buffer as negative control. Indicated values are means (n ≥ 3) ± SD.

Table 4
Pharmacokinetic data calculated after intravenous and oral administration of investigated formulations containing IG:SOS complexes in rats. Absolute bioavailability (BA%) and pharmacological activity (PA%) were calculated in reference to the i.v. injection. Indicated values are means (n = 3) ± SD.

	aqueous solution	F1	F2	i.v.
Dose (IU/kg)	85	85	85	0.5
C_{max} (mU/l)	3.7 ± 1.4	60.6 ± 59.1	58.7 ± 48.3	–
T_{max} (min)	1	90	30	–
AUC (mU/l*min)	1102 ± 107	8460 ± 2243	6821 ± 3708	9072 ± 3458
BA (%)	0.07 ± 0.01	0.55 ± 0.15	0.44 ± 0.24	100
PA (%)	0.8 ± 1.4	4.5 ± 2.3	4.3 ± 2.3	100

accelerating uptake compared to F1, consisting of Plurol® oleique CC 497 (HLB 3) as opposed to Maisine® CC (HLB 1) providing a more lipophilic core, respectively.[25–27] In addition, F2 displayed a faster increase in plasma concentration, which might be related to a higher amount of Labrasol® ALF acting as permeation enhancer (Eaimtrakarn et al., 2002; McCartney et al., 2019; Sha et al., 2005). It is necessary to consider relative bioavailability (based on plasma levels of the API) and pharmacological activity (based on the API effect) separately when evaluating pharmacokinetic data. For example, Liu et al. reached a relative bioavailability of 0.11%, whereas Bashyal et al. achieved 6.44% pharmacological activity utilizing 100% Labrasol® ALF and bile salt transport pathways with both groups utilizing insulin intrajejunal administration (Bashyal et al., 2021; Liu et al., 2019). Despite administering the SEDDS formulations via oral gavage, so potentially exposing IG to gastric related degradation and reprecipitation at intestinal pH, comparable (PA) or even higher (BA) results were obtained within this study, with a PA and BA of 4.5% and 0.55% for F1, respectively. Therefore, within this study, IG was for the first time complexed via hydrophobic ion pairing and incorporated into a SEDDS formulation, achieving increased lipophilicity and successful oral administration in vivo.

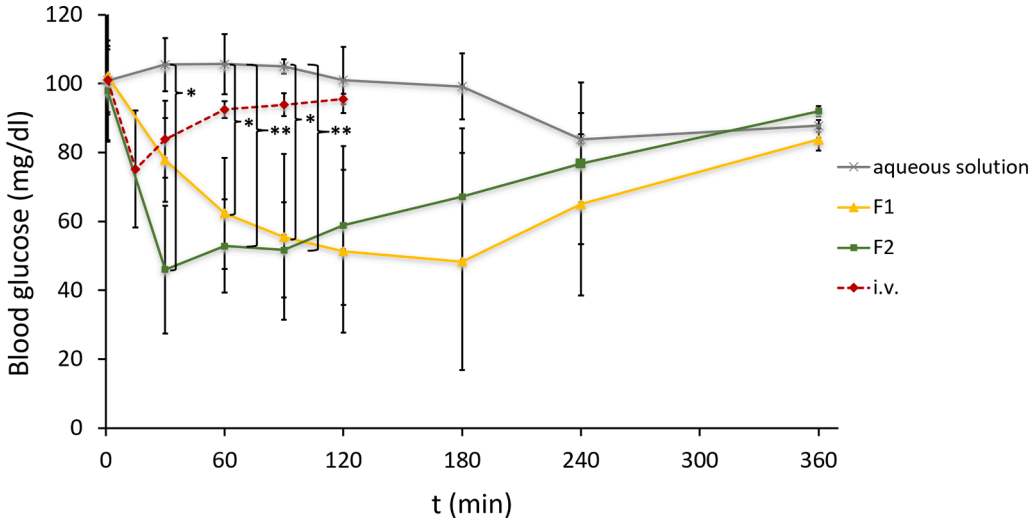


Fig. 7. Blood glucose level after oral administration of IG:SOS SEDDS formulations F1 (▲), F2 (■) and aqueous IG solution (-x-) (dose: 85 U/kg bodyweight)) as well as i.v. IG solution (-◆-) (dose: 0.5 U/kg). Indicated values are means ($n \geq 3$) ± SD. Significant differences are indicated with * ($p < 0.05$), very significant differences are indicated with ** ($p < 0.01$).

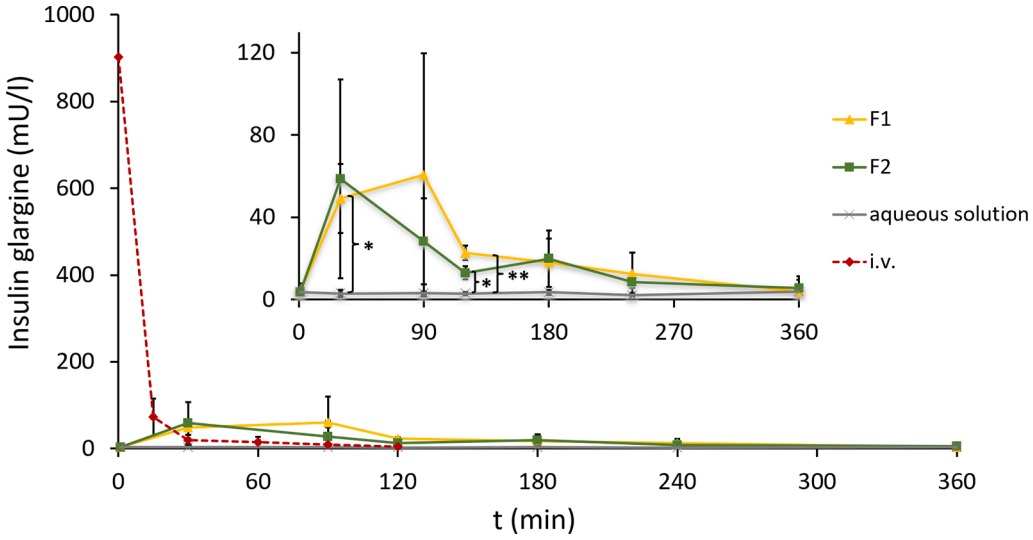


Fig. 8. Insulin glargine (IG) plasma concentration–time curve for orally administered IG:SOS SEDDS formulations F1 (▲), F2 (■) and aqueous IG solution (-x-) (dose: 85 U/kg) as well as i.v. IG solution (-◆-) (dose: 0.5 U/kg). Indicated values are means ($n \geq 3$) ± SD. Significant differences are indicated with * ($p < 0.05$), very significant differences are indicated with ** ($p < 0.01$).

4. Conclusion

In this study, insulin glargine (IG) was investigated as a possible alternative to unmodified insulin for hydrophobic ion pairing. Highly lipophilic complexes were successfully formed using sodium octadecyl sulfate, enabling subsequent incorporation into lipid-based nanocarriers. Self-emulsifying drug delivery systems (SEDDS) were then developed with a lipophilic core comprising Labrasol® ALF as permeation enhancer to improve uptake in vivo. Hemolysis and cell viability assays indicated safety levels suitable for in vivo studies. Due to the high lipophilicity of the complex, sufficient log $D_{SEDDS/release\ medium}$ values were achieved, which is considered essential for in vivo efficacy. Indeed, oral administration of developed drug delivery systems F1 and F2 resulted in a 7.7- and 6.2-fold improved uptake of IG, respectively, providing proof-of-concept for the oral delivery of peptide drugs utilizing SEDDS technology. In order to further increase bioavailability a higher lipophilic character of the complex formed between insulin and hydrophobic counter ions is likely advantageous. It will provide higher payloads, keep the drug to a higher extent in the oily droplets and will likely improve membrane permeability. The design of new more suitable counter ions such as more potent analogues of docusate will contribute to this strategy (Wibel et al., 2023).

CRediT authorship contribution statement

Victor Claus: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Helen Spleis:** Investigation. **Christoph Federer:** Investigation. **Katrin Zöller:** Investigation. **Richard Wibel:** Investigation. **Flavia Laffleur:** Investigation. **Camille Dumont:** Project administration. **Philippe Caisse:** Project administration. **Andreas Bernkop-Schnürch:** Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal

relationships which may be considered as potential competing interests: Andreas Bernkop-Schnuerch reports financial support was provided by Gattefossé SAS. Camille Dumont and Philippe Caisse report a relationship with Gattefossé SAS that includes: employment.

Data availability

The data that has been used is confidential.

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