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Development and *in vivo* evaluation of nanoemulsions for oral delivery of low molecular weight heparin

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ABSTRACT

Aim: The aim of this study was the development and *in vivo* evaluation of different types of nanoemulsions for oral delivery of low molecular weight heparin (LMWH).
Methods: Nanoemulsions (NE) were developed consisting of mainly non-digestible components and variations of polyglycerol-, PEGylated or zwitterionic surfactants. To incorporate LMWH in the oily droplets, hydrophobic ion pairs were formed with several cationic surfactants. Loaded and unloaded NE were evaluated for their size, zeta potential, stability towards pancreatin, hemolytic and toxic properties. Finally, an *in vivo* study on oral LMWH delivery was performed.
Results: Droplet size of all formulations was below 150 nm with a PDI <0.4. Highest $\log K_{1\text{-butanol/water}}$ was obtained for the complex of LMWH and didecyldimethylammonium bromide (HIP). This complex was incorporated into NE containing polyglycerol- and PEGylated surfactants up to a concentration of 5% (m/v) and in zwitterionic NE up to 10% (m/m). NE containing polyglycerol-surfactants were digested to a higher extent than NE containing PEGylated surfactants. The zwitterionic NE without glycerides did not show any lipolysis but the zwitterionic NE which contained high amounts of glycerides was rapidly degradable. NE with polyglycerol-surfactants were less toxic and hemolytic than NE with PEGylated surfactants and zwitterionic NE. Toxic potential increased with HIP-loaded formulations. When performing *in vivo* studies, oral bioavailability of LMWH was increased up to 2.6% with non-digestible NE.
Conclusion: According to these results, non-digestible nanoemulsions improve oral bioavailability of LMWH more efficiently than biodegradable formulations.

1. Introduction

In recent decades, lipid-based nanocarriers like liposomes, nanoemulsions and self-emulsifying drug delivery systems have received great attention for oral drug delivery as they could provide an alternative to parenteral administration due to increased solubility, stability and absorption of drugs within the gastrointestinal tract [1]. Hydrophilic macromolecular drugs such as therapeutic peptides or polysaccharides are inactivated within the gastrointestinal tract or can not overcome the mucus and epithelial barrier to reach systemic circulation [2–4]. Incorporating these drugs into lipid-based nanocarriers can protect them from enzymatic degradation and enhance permeation [1,5]. In order to incorporate these hydrophilic drugs into the lipid droplets, lipophilicity has to be increased via hydrophobic ion pairing with counterions [2,6]. As long as the complex remains intact, the hydrophobic ion pair can be absorbed from the intestine. Disintegration of the complex is either mediated by counterions, such as electrolytes or bile salts, or pH-driven [6]. Since these competing endogenous counterions or acids are too hydrophilic to enter the lipid formulation, the complex remains intact as long as it is incorporated within these oily droplets. This concept works consequently only if the drug is not released from the droplets. Enzymes present in the gastrointestinal tract are able to degrade these lipid-based nanocarriers which could lead to undesired drug release [7,8]. Non-degradable lipid-based nanocarriers are therefore likely the better choice for oral administration of these drugs. Furthermore, surface decoration has an impact on their biodegradability

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Table 1
Composition of NE containing polyglycerol- and PEGylated surfactants in % (v/v) and of zwitterionic NE in % (m/m). Size [nm], PDI and zeta potential [mV] of NE in a dilution of 1% in water measured at 37 °C. logD of LMWH didecyldimethylammonium between NE preconcentrate and FaSSIF. Data are presented as means ± standard deviation.

	% (v/v)	size [nm] 1% in water 37 °C	PDI	zeta potential [mV]	logD NE/FaSSIF
NE PG					
Polyglyceryl-4 caprate	30	133.5 ± 20.3	0.355 ± 0.039	−22.9 ± 0.4	2.63 ± 0.22
Polyglyceryl-4 laurate/sebacate (and) polyglyceryl-6 caprylate/caprate (and) aqua	20				
Oleyl alcohol	20				
Isopropyl myristate	30				
NE PEG PG I					
Polyglyceryl-3 caprylate/caprate/succinate (and) propylene glycol	30	76.6 ± 7.4	0.159 ± 0.014	−4.8 ± 1.0	2.41 ± 0.05
PEG-35 castor oil	20				
Oleyl alcohol	20				
Isopropyl myristate	30				
NE PEG					
PEG-35 castor oil	50	54.5 ± 2.9	0.320 ± 0.045	−7.0 ± 0.3	2.62 ± 0.13
Oleyl alcohol	20				
Isopropyl myristate	30				
NE PEG PG II					
Polyglyceryl-3 caprylate/caprate/succinate (and) propylene glycol	20	130.2 ± 15.2	0.215 ± 0.029	1.2 ± 0.9	4.06 ± 0.09
PEG-35 castor oil	20				
Oleyl alcohol	30				
Octanol	30				
	% (m/m)	**size [nm]** 1% in water 37 °C	PDI	zeta potential [mV]	**logD** NE/FaSSIF
NE Z1					
Lysophosphatidylcholine	45	97.1 ± 1.9	0.312 ± 0,046	−6.95 ± 4.21	/
Oleyl alcohol	20				
Isopropyl myristate	35				
NE Z2					
Lysophosphatidylcholine	45	52.4 ± 8.7	0.310 ± 0.009	−7.65 ± 0.35	/
Cholesterol	10				
Medium chain triglycerides	15				
Glyceryl caprylate/caprate Type I	30				

and cellular uptake. For instance, PEGylated surfactants form an inert, hydrophilic polymer layer around the carriers [9] and PEGylation sterically inhibits adsorption of lipase [10] and consequently degradation is reduced. These favourable properties, however, cause low cellular uptake [9,11]. Replacing PEGylated with polyglycerol-surfactants seems to address this problem, as although polyglycerol surfaces form an inert, hydrophilic layer around carriers, cellular uptake is comparatively high [9,11,12]. Although polyglycerol-surfactants might be advantageous in terms of permeation properties, the ester between glycerol and fatty acids within the surfactant could be easily cleaved by pancreatic lipase causing instability of the nanocarrier. Therefore, a combination of both surfactants within a formulation might combine the features of low digestibility and increased cellular uptake. Another strategy for enhanced uptake could be zwitterionic surfaces which are inspired by viruses and provide special hydration behaviour to efficiently overcome the mucus layer [2,13]. Phospholipids can imitate these zwitterionic surfaces but can be cleaved by enzymes like phospholipase A_2 present in pancreatin [14].

The aim of this study was to investigate the influence of different nanoemulsions (NE) varying in composition of polyglycerol-, PEGylated or zwitterionic surfactants on oral LMWH bioavailability. LMWH, which is a commonly used anticoagulant for the prevention of arterial and venous thrombosis [3,15], was chosen as a model drug due to its high molecular weight and hydrophilicity being responsible for its poor oral bioavailability [3,16–18]. Several research groups have already focused on strategies to increase oral bioavailability of LMWH including hydrophobic ion pairing [18,19], nanoparticles [4] and the use of absorption enhancers [16,20–24]. In this study, NE with mainly non-digestible excipients and varying surfactants namely polyglycerol-, PEGylated and zwitterionic surfactants were assessed for their extent of digestion mediated by pancreatin. Since LMWH is a highly hydrophilic drug, its lipophilicity had to be increased via hydrophobic ion pairing with cationic surfactants for incorporation into lipid droplets. Additionally, loaded and unloaded formulations were investigated for their size, PDI as well as hemolytic and toxic potential. Finally, the most promising formulations considering payload and droplet size were assessed in an *in vivo* study.

2. Materials and methods

2.1. Materials

LMWH (Enoxaparin Becat 4000 I.E. 40 mg/0.4 ml) was purchased from Rovi GmbH (Holzkirchen, Germany). DOTAP chloride and Lipoid P LPC 80 (lysophosphatidylcholine from soybean, 80%) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Imwitor 742® (medium chain partial glycerides, glyceryl caprylate/caprate Type I) was purchased from IOI Oleo GmbH (Hamburg, Germany) and didecyldimethylammonium bromide from Tokyo Chemical Industry Co. (Tokyo, Japan). Miglyol® 812 (medium chain triglycerides) was bought from Caesar & Loretz GmbH (Bonn, Germany) and sodium taurodeoxycholate hydrate (NaTDC) was bought from Biosynth (Bratislava, Slovakia). Dodecylamine HCl, dodecyltrimethylammonium chloride and cholesterol were obtained from ThermoFisher GmbH (Kandel, Germany). Tegosoft® PC41 (polyglyceryl-4 caprate) and Tego® solve 55 (polyglyceryl-3 caprylate/caprate/succinate (and) propylene glycol) were kindly provided by Evonik (Hamburg, Germany) as free samples. Natragem™ S140 NP (polyglyceryl-4 laurate/sebacate (and) polyglyceryl-6 caprylate/caprate (and) aqua) was supplied by Croda Inc. (Nettetal, Germany). FaSSIF powder was obtained from Biorelevant (London, United Kingdom). Pancreatin from porcine pancreas [8x USP specifications], L-alpha-phosphatidylcholine from egg yolk [60%] (PC), Kolliphor EL® (PEG-35 castor oil) and all other chemicals were purchased from Sigma-Aldrich (Vienna, Austria) or other commercial sources.

2.2. Size exclusion HPLC (SEC-HPLC)

SEC-HPLC was utilized for quantification of LMWH as described by Matanovic et al. [25] with some minor modifications. The HPLC system consisted of a Merck Hitachi Elite LaChrom HPLC-system with a L-2130 pump, L-2200 autosampler and L-2450 diode array detector (Vienna, Austria). A BioSep-SEC-S 2000 column (300 × 7.8 mm, 5 μm) (Aschaffenburg, Germany) was used as stationary phase and 1 mg/ml arginine solution (pH 6.5) as mobile phase. Samples (90 μl) were injected and detected at 215 nm [19]. All samples were measured at room temperature.

2.3. Hydrophobic ion pairing between LMWH and cationic surfactants

LMWH solution was diluted to a final concentration of 2 mg/ml. Surfactant solutions were prepared in molar ratios from 5:1 to 40:1 (surfactant:LMWH). The surfactants dodecyltrimethylammonium

chloride and didecyldimethylammonium bromide were dissolved in demineralized water. Dodecylamine HCl was dissolved in 0.01 M HCl to increase its cationic character. A mixture of ethanol and water (1:1) was used for preparation of the DOTAP chloride and tetraheptylammonium bromide solution. Thereafter, 500 µl of surfactant solution was added dropwise to 500 µl of LMWH solution. The mixtures were incubated on a Thermomixer (Eppendorf ThermoMixer® C, Eppendorf AG, Germany) at 25 °C and 400 rpm for 2 h. Afterwards, the samples were centrifuged with a MiniSpin® (Eppendorf AG, Germany) at 12500 rpm for 10 min [26,27]. Remaining LMWH in the supernatant was quantified by SEC-HPLC as described above. The precipitated LMWH-surfactant complex was washed twice with demineralized water and lyophilized (Christ Gamma 1–16 LSC Freeze Dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Germany)) [19]. The complex was then stored at room temperature. Complex formation was calculated by the following equation:

$$Complex\ formation\ [\%] = 100 - \left(\frac{LMWH\ concentration\ after\ HIP}{LMWH\ concentration\ before\ HIP} \times 100\right)$$

2.4. $logK_{1\text{-}butanol/water}$ determination

LMWH-surfactant complexes were dispersed in a biphasic system consisting of 500 µl of 1-butanol and 500 µl of demineralized water. 1-Butanol was used instead of 1-octanol because not all complexes were soluble in 1-octanol [27]. The samples were incubated at 37 °C and 300 rpm in a Thermomixer for 24 h. After 24 h, samples were centrifuged at 12500 rpm for 10 min and phases were separated. For SEC-HPLC, 100 µl of the butanol phase was diluted with 300 µl of methanol [26] whereas the aqueous phase was analysed without further dilution. LMWH was quantified in both phases via SEC-HPLC. When LMWH concentrations in the aqueous phase were below the detection limit, Azure A assay was used for quantification. For the Azure A assay, equal volumes of Azure A solution (80 µmol/l) and aqueous phase were mixed and absorbance was measured at 512 nm [25]. logK was calculated using the following equation:

$$logK_{1-butanol/water} = log\frac{c_{LMWH\ in\ 1-butanol}}{c_{LMWH\ in\ water}}$$

2.5. Preparation of nanoemulsions

For NE preconcentrate preparation, excipients as listed in Table 1 were homogenized at 37 °C and 1250 rpm in a Thermomixer for 24 h. NE preconcentrates containing lysophosphatidylcholine were blended at 80 °C. The hot NE preconcentrates were emulsified in a ratio of 1:100 in demineralized water for further experiments. Since NE containing lysophosphatidylcholine formed droplets with sizes >500 nm, samples were homogenized via ultrasonication (UP 200H, Hielscher Ultrasonics GmbH, Germany) at an ultrasonic frequency of 24 kHz and an amplitude of 100% for 1 min to form droplets below 500 nm.

2.6. Characterization of nanoemulsions

NE 1% in water were characterized for their size, polydispersity index (PDI) and zeta potential. Parameters were determined via dynamic light scattering using a Zetasizer Nano ZSP (Malvern Panalytical Ltd., United Kingdom). NE were briefly vortexed for emulsification and preheated to 37 °C before the measurement [9]. NE containing lysophosphatidylcholine were additionally ultrasonicated before the measurements as described above. All measurements were executed at 37 °C to simulate *in vivo* conditions.

Furthermore, logD of the hydrophobic ion pair LMWH didecyldimethylammonium (HIP) between NE preconcentrate and fasted state simulated intestinal fluid (FaSSIF) was determined by quantifying the maximum solubility of HIP in the organic and aqueous phase [28]. Therefore, a surplus of HIP was added to 100 µl of NE preconcentrate or to 100 µl of FaSSIF and incubated at 37 °C and 1200 rpm on a Thermomixer for 24 h. FaSSIF was prepared according to the supplier's manual. Afterwards, the samples were centrifuged at 12500 rpm for 10 min. NE preconcentrate was diluted with methanol and LMWH was quantified via SEC-HPLC. In case of interfering oil peaks in the HPLC chromatogram, samples were diluted with water and quantified via Biophen® Heparin Anti-Xa assay utilizing the manual method. LMWH concentration in FaSSIF was determined directly without further dilution. LogD was then calculated via the maximum LMWH solubility in both media [29,30]:

$$logD_{NE\ preconcentrate/FaSSIF} = log\frac{c_{LMWH\ in\ NE\ preconcentrate}}{c_{LMWH\ in\ FaSSIF}}$$

Moreover, stability of NE 1% at 37 °C was evaluated. For this reason, NE 1% were prepared as described above and incubated at 37 °C. Additionally, stability of NE 15 was investigated in FaSSIF and fasted state simulated gastric fluid (FASSGF) having been prepared according to the supplier's manual. Size and PDI were measured with the Zetasizer Nano ZSP at designated time points over a time period of 7 h [26].

2.7. Payload

HIP was incorporated in concentrations of 0.5%, 1%, 2.5%, 5% and 10% in the NE preconcentrates (m/v) at 37 °C and 1250 rpm in a Thermomixer for 24 h. Samples were centrifuged at 12500 rpm for 10 min and visually investigated for homogeneity and precipitation [26]. Additionally, the payload was confirmed after dilution of preconcentrate with methanol or water via SEC-HPLC or Biophen® Heparin Anti-Xa assay.

Furthermore, NE preconcentrates with increasing amounts of incorporated HIP were diluted in a ratio of 1:100 in demineralized water, vortexed and heated up to 37 °C. NE containing lysophosphatidylcholine in a concentration of 1% were additionally ultrasonicated. Samples were evaluated for size, PDI and zeta potential with the Zetasizer Nano ZSP at 37 °C.

2.8. Lipolysis studies

Digestion buffer was prepared by dissolving 150 mM NaCl, 1.4 mM $CaCl_2{\cdot}H_2O$, 2 mM Tris-maleate, 3 mM NaTDC and 0.75 mM PC in demineralized water [7]. To assure complete dissolution of PC, the medium was stirred for at least 4 h before adjustment of pH to 6.5. For the enzyme solution, 1 g of pancreatin was suspended in 5 ml of cold (2–8 °C) digestion buffer (without NaTDC and PC). To avoid loss in enzymatic activity, the solution was freshly prepared before each experiment. After 10 min, the suspension was centrifuged at 12500 rpm and 4 °C for 10 min (Sigma 3-18 KS, Sigma Laborzentrifugen GmbH, Germany). The clear supernatant was collected for the following experiment and pH was adjusted to 6.5 with approximately 50 µl of 1 M NaOH.

Lipolysis studies were performed as described by Williams et al. [7] with some modifications. Briefly, 1 g of polyglycerol-surfactants or 400 µl of NE preconcentrate was emulsified and if necessary ultrasonicated in 36 ml of digestion buffer. The emulsion was stirred at 37 °C for 10 min and pH was adapted to 6.5 ± 0.05 before starting the lipolysis experiment. By addition of the enzyme solution lipolysis was initiated. Enzymes in pancreatin lead to a release of fatty acids which decreased the pH. Titration with 1 M or 0.1 M NaOH took place at designated time points over a time period of 60 min to lift pH back to 6.5. Additionally, digestion experiments solely of the digestion medium were carried out due to possible hydrolysis of PC, impurities in the bile salts or pancreatic extract [31]. These values were subtracted from the results of the original samples.

Additionally, back titrations were performed after 60 min to detect the only partially ionized fatty acids. Therefore, pH was increased

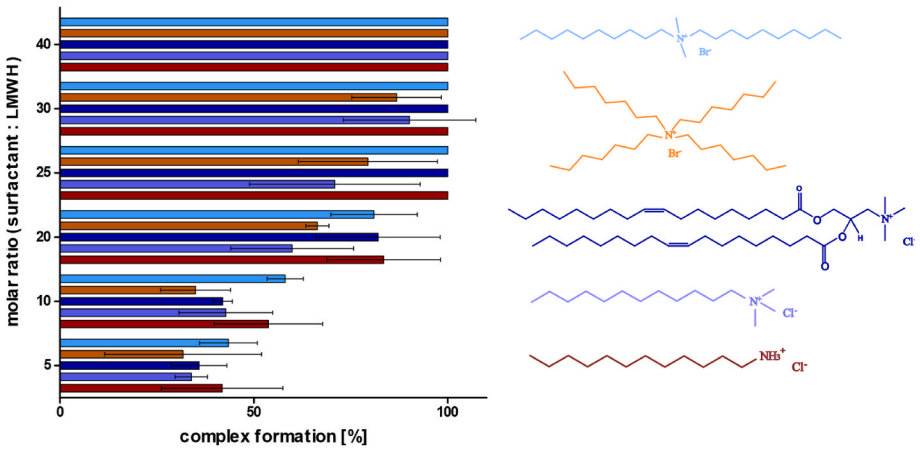

Fig. 1. Complex formation [%] between LMWH and the cationic surfactants dodecylamine HCl (dark red), dodecyltrimethylammonium chloride (purple), DOTAP chloride (dark blue), tetraheptylammonium bromide (orange) and didecyldimethylammonium bromide (light blue) in ascending molar ratios (surfactant:LMWH). Data are presented as means ± standard deviation. Structures of the cationic surfactants are shown on the right. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

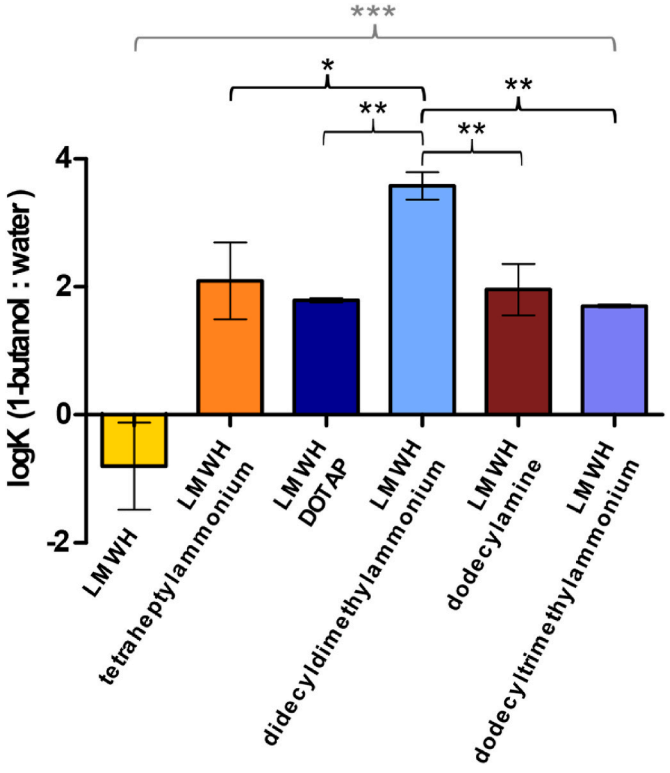

Fig. 2. $\log K_{1\text{-butanol/water}}$ of LMWH and complexes between LMWH and the cationic surfactants tetraheptylammonium bromide (orange), DOTAP (dark blue), didecyldimethylammonoium bromide (light blue), dodecylamine HCl (dark red) and dodecyltrimethylammonium chloride (purple). Data are presented as means ± standard deviation. Significant differences are indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Grey bracket represents significant difference (***) between LMWH and all complexes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

rapidly to 9 with 1 M NaOH at the end of each lipolysis experiment [32]. Blank experiments were performed as well and subtracted from the original results.

2.9. Hemolysis assay

In vitro hemolysis of NE and HIP-loaded NE was investigated by evaluating the hemolytic activity on erythrocytes concentrate which was kindly donated by Tirol Kliniken GmbH (Innsbruck, Austria).

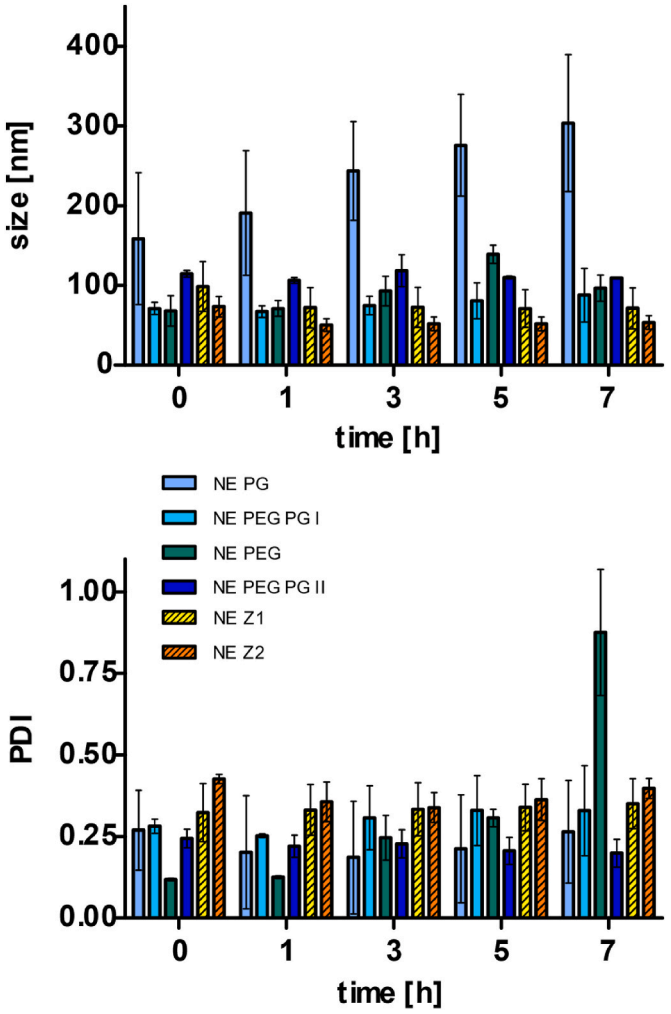

Fig. 3a. Alterations of size [nm] and PDI of NE 1% in water at 37 °C over 7 h. Bars represent *NE PG* (light blue), *NE PEG PG I* (turquoise), *NE PEG* (dark green), *NE PEG PG II* (dark blue), *NE Z1* (yellow) and *NE Z2* (orange). Data are presented as means ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Hemolytic effects of aqueous LMWH solution and HIP dissolved in DMSO/glucose-HEPES buffer (5:95) were investigated as well. Erythrocytes concentrate was suspended in a ratio of 1:200 with sterile

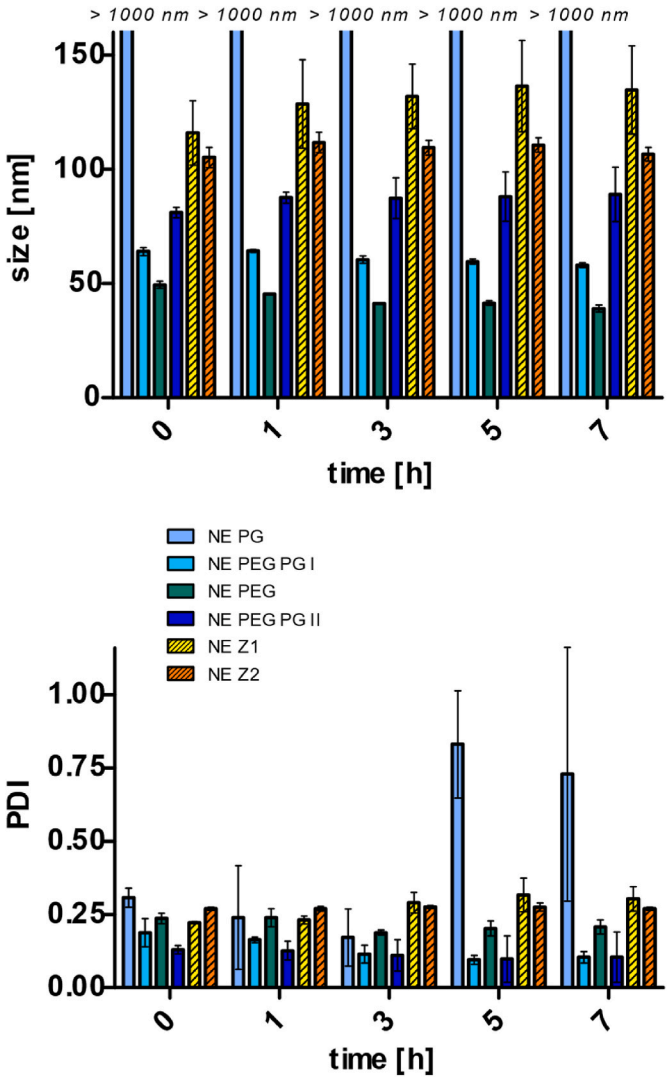

Fig. 3b. Alterations of size [nm] and PDI of NE 1% in FaSSGF at 37 °C over 7 h. Bars represent *NE PG* (light blue), *NE PEG PG I* (turquoise), *NE PEG* (dark green), *NE PEG PG II* (dark blue), *NE Z1* (yellow) and *NE Z2* (orange). Data are presented as means ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

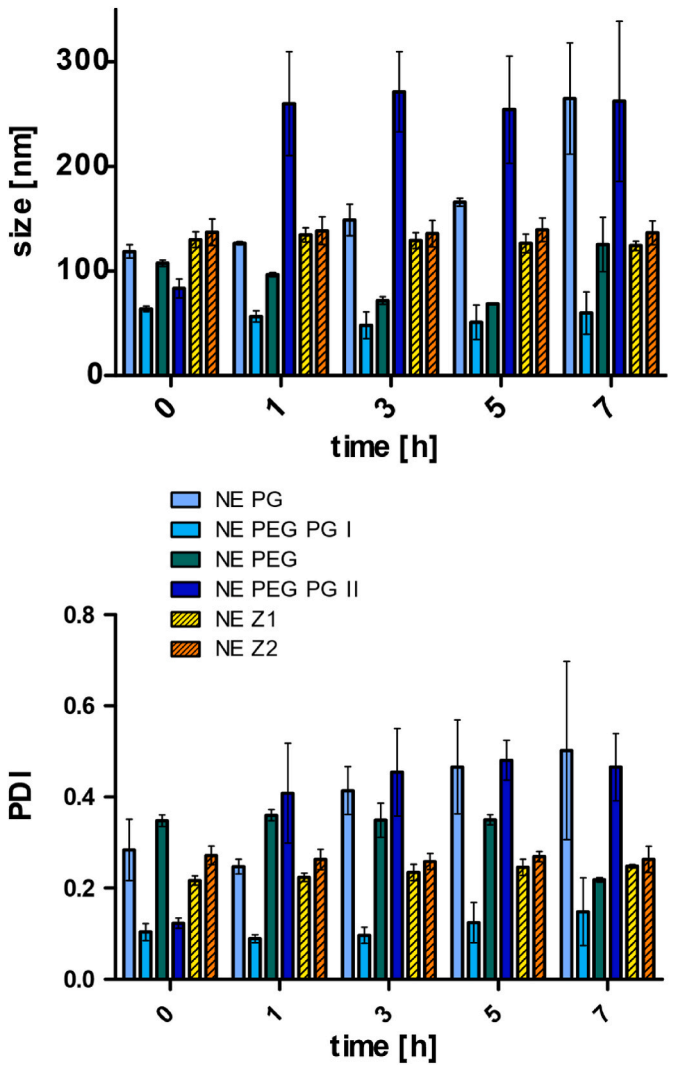

Fig. 3c. Alterations of size [nm] and PDI of NE 1% in FaSSIF at 37 °C over 7 h. Bars represent *NE PG* (light blue), *NE PEG PG I* (turquoise), *NE PEG* (dark green), *NE PEG PG II* (dark blue), *NE Z1* (yellow) and *NE Z2* (orange). Data are presented as means ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

glucose-HEPES buffer of pH 7.4. 500 µl of emulsified NE in glucose-HEPES buffer was added to an equal volume of diluted erythrocyte suspension. Resulting concentrations of NE were 0.005%, 0.01%, 0.05% and 0.1% (v/v). Samples were incubated in an Orbital Shaker Incubator ES-80 (Grant Instruments Ltd, United Kingdom) at 150 rpm and 37 °C for 4 h. Thereafter, samples were centrifuged at 2700 rpm for 10 min and 100 µl of the supernatant was quantified for released hemoglobin via UV-spectrometry at a wavelength of 415 nm (Tecan Spark, Tecan Sales Austria GmbH, Austria) [33]. Glucose-HEPES buffer served as negative control and 0.5% Triton-X 100 solution as positive control. The extent of hemolysis was determined by the following equation [9]:

$$Hemolysis\ [\%] = \frac{(absorption_{sample} - absorption_{negative})}{(absorption_{positive} - absorption_{negative})} \times 100$$

2.10. Cell viability – MTT assay

Potential cytotoxic effects of NE and HIP-loaded NE were investigated via the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT solution was prepared by dissolving 5 mg of MTT in 1 ml of glucose-HEPES buffer pH 7.4 with subsequent sterile filtration [34]. Right before utilization, the MTT stock solution was diluted in a ratio of 1:10 with sterile glucose-HEPES buffer. For the assay, Caco-2 cells were seeded in a density of 50 000 cells per well and incubated for three 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 [9]. After three days, MEM was removed and cells were washed twice with 100 µl sterile glucose-HEPES buffer pH 7.4 before addition of 100 µl of NE samples. Tested concentrations of NE and HIP-loaded NE were 0.025%, 0.05% and 0.1% (v/v). Additionally, aqueous LMWH solution and HIP dissolved in DMSO/glucose-HEPES buffer (5:95) were assessed for their toxic potential. Sterile glucose-HEPES buffer and 0.1% Triton-X 100 served as negative and positive control for cytotoxic effects. Afterwards, the plate was incubated at 37 °C for 24 h. After samples were removed, the cells were washed once with 100 µl of sterile glucose-HEPES buffer and 100 µl of diluted MTT solution was added. The plate was incubated light-protected at 37 °C for 2 h. Thereafter, the solution was removed carefully and formazan crystals were dissolved in 120 µl of DMSO by

Table 2
Size [nm], PDI and zeta potential [mV] of NE with increasing amount of LMWH didecyldimethylammonium (HIP) [% (m/V)]. Data are presented as means ± standard deviation.

	0.5% HIP	1% HIP	2.5% HIP	5% HIP	10% HIP
	size [nm]	size [nm]	size [nm]	size [nm]	size [nm]
	PDI zeta potential [mV]	PDI zeta potential [mV]	PDI zeta potential [mV]	PDI zeta potential [mV]	PDI zeta potential [mV]
NE PG	>1000	244.7 ± 32.3	141.9 ± 25.0	345.9 ± 65.4	>1000
	~1.000	0.351 ± 0.113	0.334 ± 0.084	0.530 ± 0.091	~1.000
	−7.3 ± 3.0	27.5 ± 1.6	30.4 ± 1.6	32.7 ± 3.7	39.8 ± 2.7
NE PEG PG I	66.4 ± 3.8	65.7 ± 2.2	87.5 ± 4.1	139.1 ± 6.1	250.8 ± 21.8
	0.264 ± 0.055	0.279 ± 0.046	0.353 ± 0.064	0.312 ± 0.051	0.402 ± 0.032
	8.0 ± 1.7	12.6 ± 2.4	27.7 ± 0.1	35.0 ± 3.2	39.2 ± 1.3
NE PEG	148.0 ± 50.5	116.3 ± 37.3	320.7 ± 87.4	761.7 ± 176.1	>1000
	0.217 ± 0.043	0.314 ± 0.228	0.341 ± 0.140	0.719 ± 0.202	~1.000
	1.1 ± 1.2	9.1 ± 4.1	9.3 ± 1.3	10.6 ± 2.2	17.3 ± 1.9
NE PEG PG II	148.7 ± 19.5	108.3 ± 2.1	101.6 ± 1.9	194.7 ± 30.3	687.5 ± 378.6
	0.273 ± 0.017	0.249 ± 0.009	0.286 ± 0.023	0.389 ± 0.061	0.719 ± 0.208
	15.8 ± 2.0	21.1 ± 2.4	32.4 ± 6.6	48.0 ± 2.0	53.1 ± 2.7
NE Z1	>1000	>1000	414.5 ± 42.3	196.3 ± 9.8	174.2 ± 9.5
	~1.000	~1.000	0.133 ± 0.006	0.187 ± 0.011	0.226 ± 0.006
	0.5 ± 0.6	−0.4 ± 0.8	9.3 ± 1.5	27.5 ± 1.4	41.3 ± 1.8
NE Z2	>1000	>1000	218.9 ± 6.7	143.0 ± 18.1	163.5 ± 11.0
	~1.000	~1.000	0.116 ± 0.013	0.229 ± 0.005	0.210 ± 0.006
	2.4 ± 0.2	3.2 ± 1.4	11.8 ± 0.8	28.3 ± 1.5	37.1 ± 0.8

placing it in the orbital shaker incubator at 150 rpm for 10 min. Finally, 100 µl of the solution was withdrawn and measured spectrophotometrically at 570 nm with the Tecan Spark [35].

2.11. In vivo studies

In vivo studies were executed according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and were approved by the Animal Ethical Committee of Vienna, Austria (GZ: 2022–0.792.904). Male Sprague-Dawley rats with a mean body weight between 250 and 350 g were supplied by Janvier Labs (Saint Berthevin, France). Rats were randomly divided into four groups (n = 3) and had free access to water and food during the experiment. The first group received intravenously 100 µl of aqueous LMWH solution (0.2 mg/kg). The other groups received 500 µl of diluted HIP-loaded *NE PEG PG II* with a dosage of 10 mg/kg LMWH and 400 mg/kg NE or *NE Z1/2* with a dosage of 20 mg/kg LMWH and 400 mg/kg NE via oral gavage. Different dosages of LMWH were chosen according to the maximum payload of the NE with acceptable droplet sizes and PDI investigated in 3.3 Incorporation of HIP in nanoemulsions. Blood samples were collected from the tail vein at designated time points over 6 h. To prevent clotting, the blood samples were immediately mixed with 20 µl of 3.8% sodium citrate and centrifuged at 6000 rpm for 20 min. The plasma was separated and stored at −20 °C until analysis. LMWH in the plasma samples was quantified via Biophen® Heparin Anti-Xa kit utilizing the manual method. The linear trapezoidal rule was used for the calculation of the AUC. Absolute bioavailability was calculated using the following equation [19]:

$$Absolute\ bioavailability\ [\%] = \frac{AUC_{oral} \times dose_{i.v.}}{AUC_{i.v.} \times dose_{oral}} \times 100$$

2.12. Statistical design and analysis of data

All experiments were performed at least in triplicates and results were presented as means ± standard deviation. Statistical analysis was performed via one-way or two-way ANOVA (GraphPad Prism 5) with $p < 0.05$ considered as level of significance.

3. Results and discussion

3.1. Hydrophobic ion pairing

Incorporation of LMWH into lipid-based nanocarriers requires increased lipophilicity. For this purpose, hydrophobic ion pairing between LMWH and different cationic surfactants was carried out. LMWH is characterised by anionic glycosaminoglycans [3,15] and should therefore undergo ionic interactions with those cationic excipients [36]. Complex formation according to varying molar ratios between LMWH and the surfactants is shown in Fig. 1. Since DOTAP chloride and tetraheptylammonium bromide were insoluble in water, a 1:1 mixture of ethanol and water was utilized for the complex formation. With the exception of dodecylamine HCl, all surfactants beared quaternary ammonium groups that allowed ion pairing without pH adjustment. Complete precipitation of LMWH was achieved with all surfactants and there were no significant differences in precipitation efficiency amongst them. Charge neutralization was accomplished at a molar ratio of approximately 1:30 which is in correspondence to Eleraky et al. [18,36]. Addition of surfactant above a charge ratio between drug/surfactant 1:1 might lead to formation of micelles due to the excess of surfactant. These micelles could solubilize the precipitated complex and lower its lipophilicity [6,26,27]. In contrast to other studies [26,27,37,38], precipitation efficiency of LMWH did not decrease with increasing amounts of surfactant. When Zupancic et al. [19] performed ion pairing between LMWH and cationic surfactants, also no free LMWH was detectable in the supernatant after addition of exceeding amounts of surfactant above the predicted charge neutralization. Therefore, possibly formed micelles did not seem to facilitate dissolution of the obtained hydrophobic complexes.

To assure an increase in lipophilicity, logK between 1-butanol and water was determined (Fig. 2). 1-Butanol had to be used instead of 1-octanol due to precipitation of some complexes in 1-octanol/water [27,30]. The logK value was increased in all complexes compared to free LMWH, indicating an increase in lipophilicity. Highest logK was achieved for LMWH didecyldimethylammonium which was utilized for the following experiments. This complex consisted of 54.3% LMWH measured with SEC-HPLC after dissolution of the complex in methanol.

3.2. Nanoemulsions

NE were prepared via emulsification of the preconcentrate in

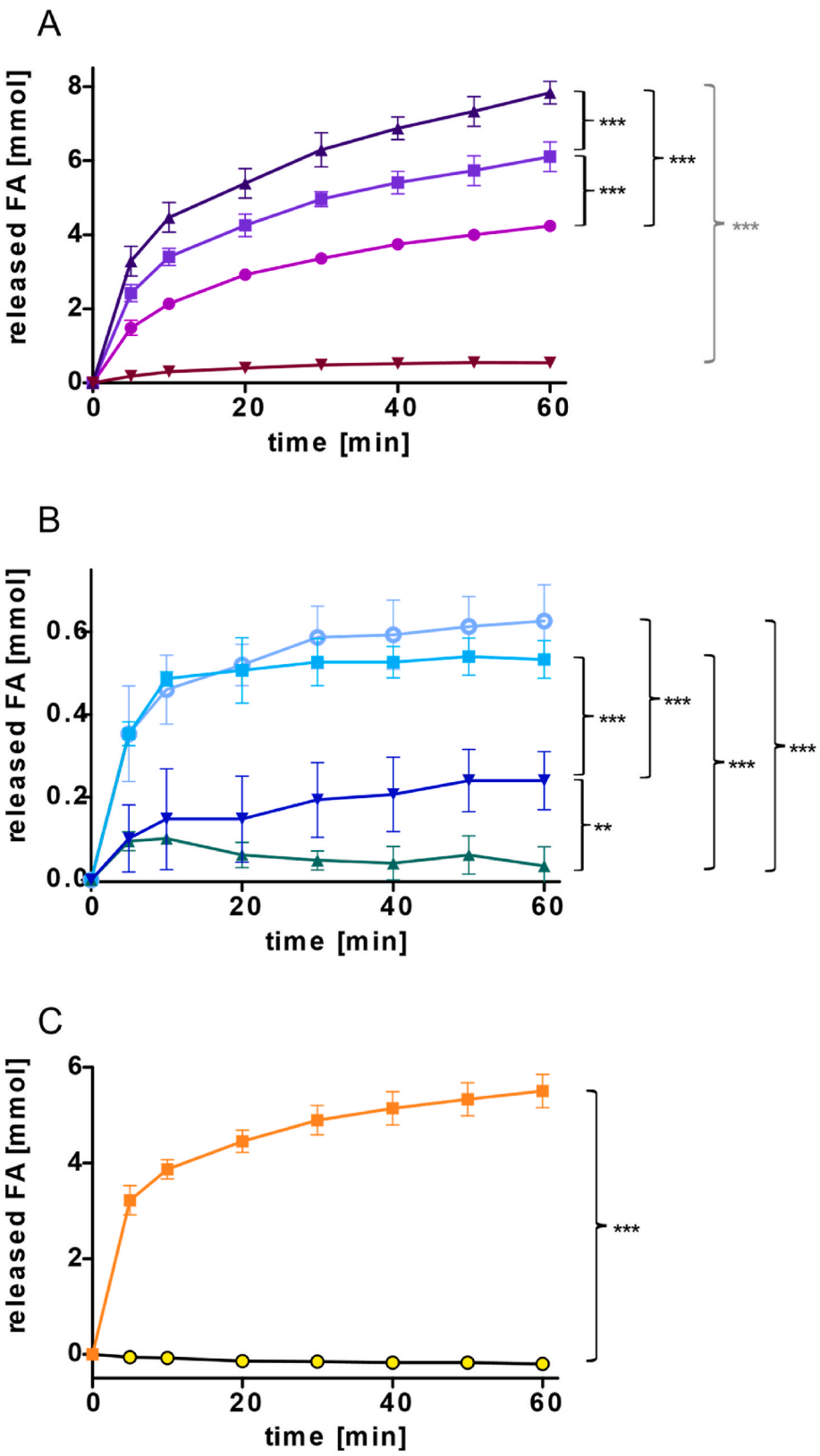

Fig. 4. Released fatty acids (FA) [mmol] of surfactants (A), NE containing polyglycerol- and PEGylated surfactants (B) and zwitterionic NE (C) after treatment with pancreatin for 60 min. A: Polyglyceryl-4 caprate (pink circles), polyglyceryl-3 caprylate/caprate/succinate (and) propylene glycol (purple squares), polyglyceryl-4 laurate/sebacate (and) polyglyceryl-6 caprylate/caprate (and) aqua (purple triangles) and lysophosphatidylcholine (dark red triangles). B: *NE PG* (light blue circles), *NE PEG PG I* (turquoise squares), *NE PEG* (green triangles) and *NE PEG PG II* (blue triangles). C: *NE Z1* (yellow circles) and *NE Z2* (orange squares). Data are presented as means ± standard deviation. Significant differences are indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Grey bracket in A represents significant difference (***) between lysophosphatidylcholine and all other surfactants. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

aqueous medium and, if necessary, ultrasonicated. To confirm the formation of NE which are basically defined as an oil-in-water or water-in-oil dispersion consisting of two immiscible liquids stabilized by suitable surfactants and droplet diameters below 500 nm [39], the formulations were evaluated for size, PDI and zeta potential. The results are shown in Table 1. Size of all formulations was below 150 nm and a PDI <0.3 indicated a narrow size distribution [40]. Formulations with a PDI above 0.3 have a broader size distribution and might not be suitable for every application. Generally, NE containing polyglycerol-surfactants were defined by greater droplet size than NE with PEGylated surfactants

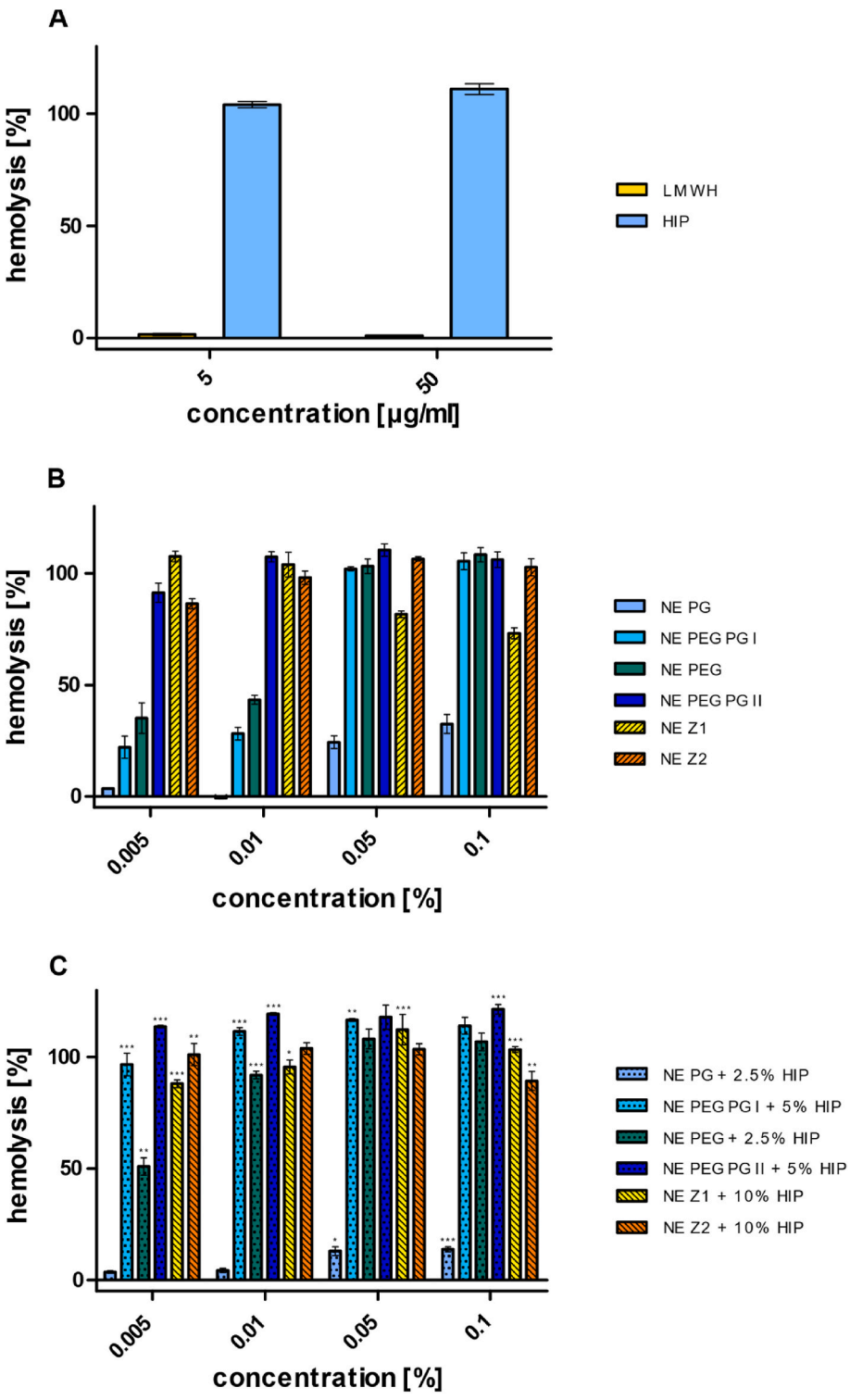

Fig. 5. Hemolysis [%] of erythrocytes after treatment for 4 h with LMWH and LMWH didecyldimethylammonium (HIP) (A), NE (B) and NE + HIP (C). A: LMWH (yellow) and HIP (blue). B: *NE PG* (blue), *NE PEG PG I* (turquoise), *NE PEG* (green), *NE PEG PG II* (dark blue), *NE Z1* (yellow) and *NE Z2* (orange). C: *NE PG* + 2.5% HIP (blue), *NE PEG PG I* + 5% HIP (turquoise), *NE PEG* + 2.5% HIP (green), *NE PEG PG II* + 5% HIP (dark blue), *NE Z1* + 10% HIP (yellow) and *NE Z2* + 10% HIP (orange). Data are presented as means ± standard deviation. Significant differences between bars of same formulation and concentration between figure B and C are indicated as $* p < 0.05$; $** p < 0.01$; $*** p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

which is in agreement with a study of Zahir-Jouzdani et al. [12]. A higher amount of polyglycerol-surfactants is required to emulsify oily droplets with the same size as emulsified with PEGylated surfactants. Surfactant to oil ratios were the same for NE PG, NE PEG PG I and NE PEG. As a result, biggest droplet size was observed for NE containing only polyglycerol-surfactants, followed by the combination of PEGylated and polyglycerol-surfactants and smallest sizes for NE comprising of PEGylated surfactants only indicating that PEGylated surfactants further support emulsification of oily hydrophobic components compared to polyglycerol-surfactants. Additionally, higher viscosity of NE preconcentrates containing polyglycerol-surfactants (Supplementary data, Fig. S1) and gelation upon contact with water contributed to longer self-emulsification time [11]. Since NE PEG PG II contained lower amounts of surfactants compared to the previously mentioned formulations, droplets were larger in size compared to NE PEG PG I. For the zwitterionic NE, addition of the preconcentrate to aqueous medium resulted as well in gelation of the formulation which impeded emulsification. Hence, these pre-formed emulsions had to be ultrasonicated to

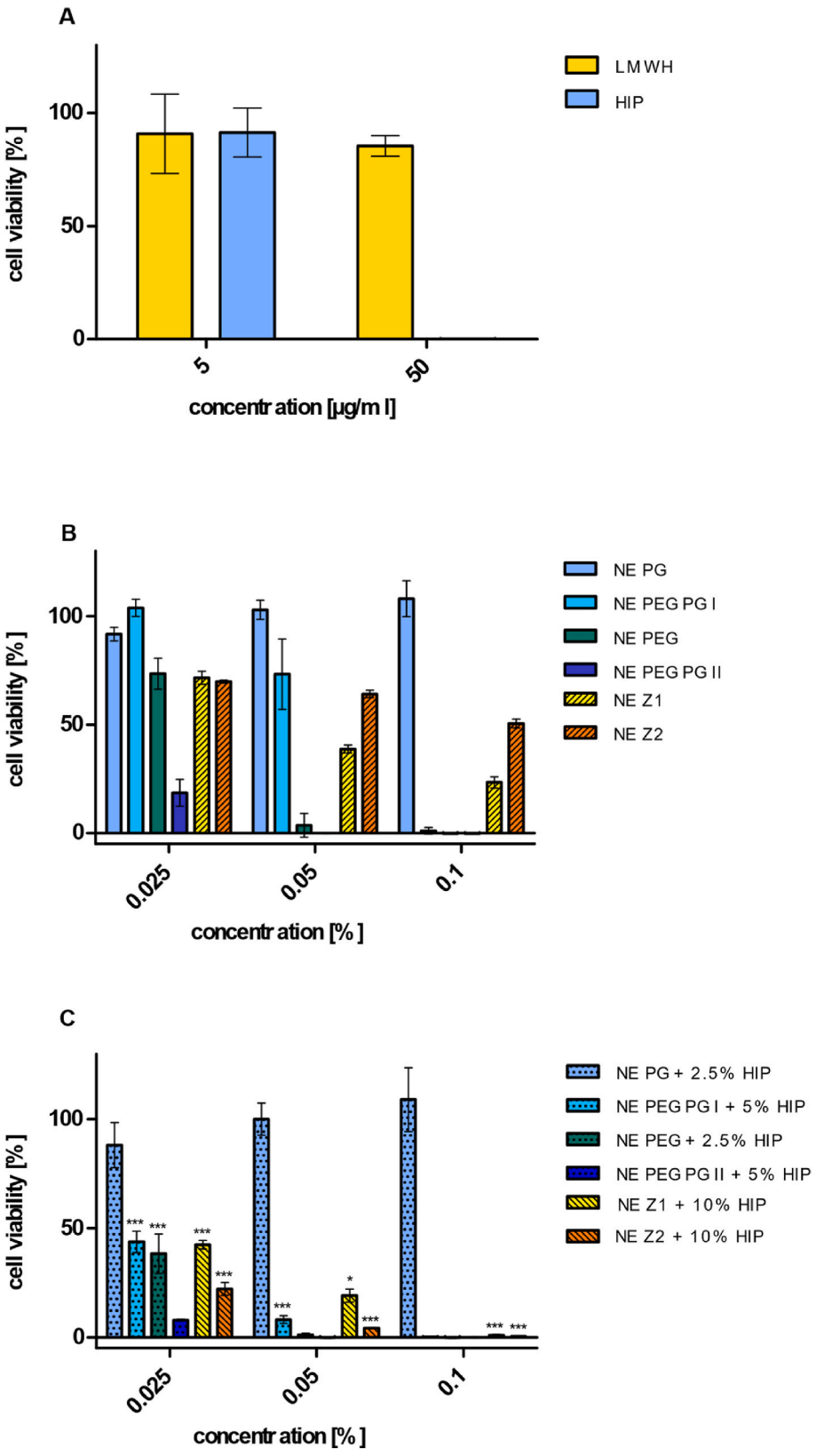

Fig. 6. Cell viability [%] of Caco2 cells after 24 h treatment with LMWH and LMWH didecyldimethylammonium (HIP) (A), NE (B) and NE + HIP (C). A: LMWH (yellow) and HIP (blue). B: *NE PG* (blue), *NE PEG PG I* (turquoise), *NE PEG* (green), *NE PEG PG II* (dark blue), *NE Z1* (yellow) and *NE Z2* (orange). C: *NE PG* + 2.5% HIP (blue), *NE PEG PG I* + 5% HIP (turquoise), *NE PEG* + 2.5% HIP (green), *NE PEG PG II* + 5% HIP (dark blue), *NE Z1* + 10% HIP (yellow) and *NE Z2* + 10% HIP (orange). Data are presented as means ± standard deviation. Significant differences between bars of same formulation and concentration between figure B and C are indicated as $* p < 0.05$; $** p < 0.01$; $*** p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

generate droplets below 500 nm. Except for *NE PG*, zeta potential was almost neutral or slightly negative. The highly negative value for *NE PG* can be explained by deprotonated carboxyl groups present in polyglyceryl-4 laurate/sebacate (and) polyglyceryl-6 caprylate/caprate (and) aqua.

To simulate the distribution of the HIP between NE and intestinal fluid, logD of HIP between NE preconcentrate and FaSSIF was determined. LogD for LMWH didecyldimethylammonium (HIP) could only be investigated for NE containing polyglycerol- and PEGylated surfactants because logD was measured at 37 °C and the zwitterionic NE

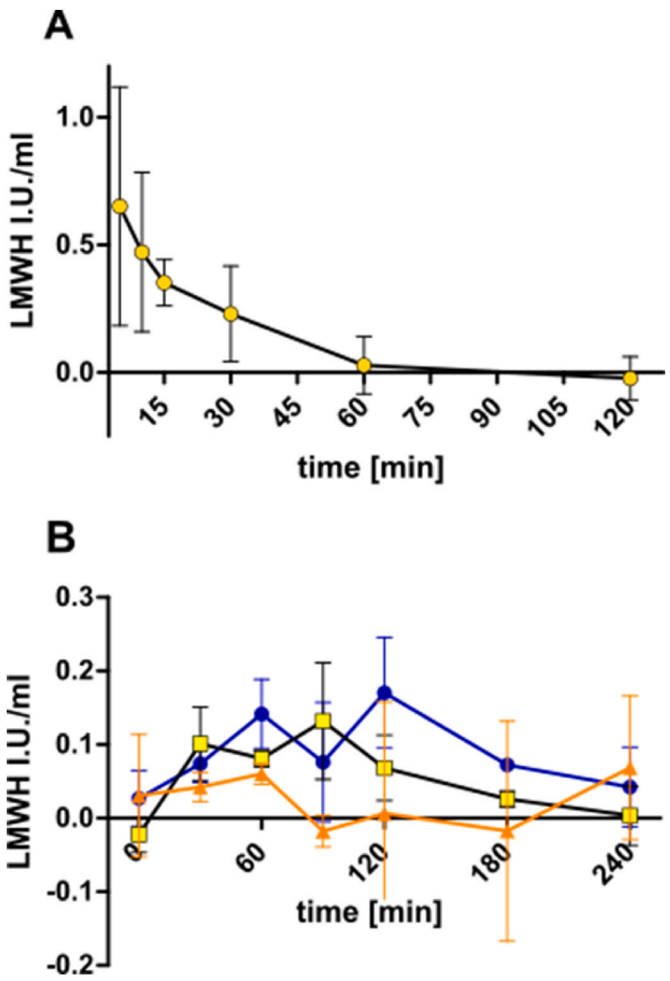

Fig. 7. Blood plasma profiles of LMWH [I.U./ml] after i.v. administration of aqueous LMWH solution (A) and oral administration of HIP-loaded NE (B). A: i.v. LMWH (yellow circles). B: *NE PEG PG II* (dark blue circles), *NE Z1* (yellow squares) and *NE Z2* (orange triangles). Data are presented as means ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Parameters of the *in vivo* study (n = 3).

	Dosage LMWH [mg/kg]	Dosage NE [mg/kg]	AUC [I.U. h/ml]	Absolute bioavailability [%]
i.v. LMWH solution	0.2	/	17.11	100
NE PEG PG II	10	400	22.40	2.6
NE Z1	20	400	19.07	0.8
NE Z2	20	400	4.25	0.2

preconcentrate was turbid and semi-solid at this temperature. Additionally, dilution with water led to precipitation of the formulation and thus quantification via Heparin Anti-Xa assay was excluded. Generally, the release of hydrophobic ion pairs from lipid-based nanocarriers is described by the diffusion from the lipid phase into the aqueous phase within a few seconds. The released ion pair is absorbed from the membrane continuously. Subsequently, more ion pair diffuses out of the lipid droplets to restore equilibrium [30,38]. Hydrophobic ion pair exhibiting a logD of 2.5 is released from oily droplets [28,41]. Assuming the dilution of 1 ml of SEDDS with 100 ml of intestinal fluid, about 25% of the drug are immediately released and permeation-enhancing effects of NE are lost. Therefore, *NE PEG PG II* seems to be the most promising formulation for further studies.

Additionally, stability of all formulations in terms of size and PDI at 37 °C was investigated over a time period of 7 h (Fig. 3a–c). Size and PDI of zwitterionic NE and *NE PEG PG* I did not change significantly within 7 h in all investigated media but sizes of the zwitterionic formulations were above 100 nm in FaSSGF and FASSIF which can be correlated to interactions with ions and other components present in the media. In water, size of *NE PG* more than doubled after 7 h although PDI did not change significantly, hence only indicating a general growth of the droplets. Increase in droplets' size of *NE PG* was also observed in FaSSIF and growing PDI might indicate degradation of droplets. In FaSSGF, droplet sizes of *NE PG* even exceeded the size of 1000 nm and PDI reached up to 1.0 within 7 h, clearly indicating that this NE was not stable within gastric environment. Ions present in FaSSGF and the low pH could have interacted with the negatively charged polyglyceryl-4 laurate/sebacate (and) polyglyceryl-6 caprylate/caprate therefore limiting droplet stability within FaSSGF. Droplets of *NE PEG* increased as well over time in water. Moreover, a PDI above 0.5 indicated high polydispersity so that partly degradation of the formulation might have occurred whereas the formulation was stable in the gastrointestinal fluids only showing a larger PDI in FaSSIF. *NE PEG PG II* revealed great stability in FaSSGF and water but interactions with components in FASSIF led to increasing size and PDI.

3.3. Incorporation of HIP in nanoemulsions

Increasing amounts of HIP were incorporated in NE preconcentrate and investigated for precipitation and homogeneity. 10% payload was achieved for all formulations. Griesser et al. [26] found comparably high payloads for peptides after hydrophobic ion pairing with anionic surfactants in SEDDS. Therefore, payload can be increased by complex formation of the hydrophilic drug with a surfactant that increases lipophilicity. Additionally, the composition of the lipid-based nanocarrier influences payload. If the complex is well soluble in the excipients used within the formulation, higher payload is likely to be obtained.

Since high loading levels might impact droplet size, the HIP-loaded formulations were evaluated for size, PDI and zeta potential (Table 2). Generally, zeta potential increased with increasing HIP incorporation which can be explained by the increasing amount of the positively charged surfactant didecyldimethylammonium bromide. High loading often leads to larger droplets [42,43]. The same effect was observed for NE investigated in this study containing polyglycerol- and PEGylated surfactants. NE consisting of a combination of polyglycerol- and PEGylated surfactants still showed a size below 200 nm and PDI <0.4 when 5% HIP was added whereas NE only composed of a polyglycerol- or PEGylated surfactant were less stable at this payload. HIP might have precipitated at higher payload and destabilized the droplets. As size and PDI of both zwitterionic NE decreased with increasing payload, HIP seemed to stabilize these formulations due to intermolecular interactions with components of the NE like lysophosphatidylcholine. Another research group investigated paclitaxel-loaded micelles [44]. Due to interactions between their formulation and the drug paclitaxel, drug-loaded micelles possessed smaller sizes than blank micelles and comparable results were found for the zwitterionic NE in this study. To assure a size <350 nm and PDI <0.4, *NE PG* and *NE PEG* were loaded with 2.5% HIP, *NE PEG PG I/II* with 5% HIP and the zwitterionic NE with 10% HIP for further experiments.

3.4. Lipolysis

Lipolysis experiments with pancreatin were performed over 60 min in order to predict the behaviour of the formulations in gastrointestinal environment. The release of fatty acids is shown in Fig. 4. It is well known that pancreatic enzymes preferably cleave ester structures [45]. Since oleyl alcohol, octanol and cholesterol do not bear any ester

structures, it was assumed that these excipients do not contribute to the release of fatty acids. Moreover, the branched isopropyl structure of isopropyl myristate sterically inhibits an enzymatic attack [46,47] and PEG-35 castor oil is only degraded to a low extent because PEGylation inhibits adsorption of lipase [10,47,48]. Therefore, polyglycerol-surfactants were mainly responsible for the digestibility of non-zwitterionic NE. Polyglycerol-surfactants are esters of fatty acids and polyglycerol. Hence, they are structurally close to the main substrates of lipase which are triglycerides [45,46]. For this reason, pancreatic enzymes should also have a high affinity to polyglycerol-surfactants [49]. This theory was proven by the digestion experiment performed within this study as all three tested polyglycerol-surfactants released large amounts of fatty acids (Fig. 4 A). Additionally, highest release of fatty acids was found for *NE PG*, followed by *NE PEG PG I/II* and least for *NE PEG*. In contrary, lysophosphatidylcholine resulted in almost no digestion. In comparison to phosphatidylcholine, where lipase and phospholipase A_2 split one ester bond [14], the remaining structure in lysophosphatidylcholine seems to be less favourable for an enzymatic attack. Moreover, phospholipids might complex with pancreatic lipase and thus inhibit adsorption of lipase to the droplet's surface and its enzymatic activity [50]. This results in almost no lipolysis for *NE Z1*. On the other hand, middle-chain glycerides present in *NE 2* led to high degradation as shown by Sek et al. [51]. Therefore, lysophopshatidylcholine, PEGylated surfactants and lipid excipients without ester structures are favoured for the design of non-digestible lipid-based nanocarriers. Although *NE PEG PG II* revealed higher lipolysis than *NE PEG*, *NE PEG PG II* was chosen for the *in vivo* study because of the obtained smaller droplets at a payload of 5%.

3.5. Hemolysis

Hemoglobin release studies are used to predict interactions between lipid-based nanocarriers and biological membranes including cytotoxicity and endosomal escape [33,52]. Hence, more hemolytic activity indicates higher endosomal escape and membrane interaction but also higher toxicity. The results for the hemolysis experiment are shown in Fig. 5. LMWH did not cause hemolysis whereas HIP resulted in 100% hemolysis. This observation might be explained by the positively charged surfactant that disrupted the membrane. Since generally the NE contained high amounts of surfactant (~50%), complete hemolysis was found for almost all formulations at a concentration of 0.1%. Additionally, even more hemoglobin was released in case of HIP-loaded formulations which might have also been caused by didecyldimethylammonium bromide. In contrary to other studies [9], NE with polyglycerol-surfactants caused less hemolysis than NE with PEGylated surfactants. An explanation might be that *NE PG*, *NE PEG PG I* and *NE PEG* consisted of the same amounts of surfactant and oils. Therefore, a direct comparison between the different surfactants and their effect on hemoglobin release was possible. Both zwitterionic NE resulted in complete hemolysis even at the lowest concentration. The NE contained lysophosphatidylcholine and other studies have already investigated its high hemolytic potential [53]. Lysophosphatidylcholine might bind to membranes in concentrations below its critical micelle concentration. Hence micelle formation does not seem to be necessary for its hemolytic activity [53]. At higher concentrations of the nanoemulsions (>0.05%), the red colour of the samples, indicating hemolysis, started to fade although there must have been complete hemolysis (data not shown). An explanation might be that the absorption spectrum of the released hemoglobin has changed since it is dependent on surfactant type, concentration and experimental conditions [54,55]. To summarise, highest toxic potential on erythrocytes was found for the zwitterionic NE and NE containing only PEGylated surfactants.

3.6. Cell viability

To assess potential toxicity, a 24 h-toxicity study on Caco-2 cells was performed (Fig. 6). Caco-2 cells can differentiate and form a monolayer with equal properties as enterocytes which are the main cells in the intestine [56]. Concentrations that provide a cell viability >80% are considered safe [11]. Hence, all unloaded formulations except *NE PEG PG II* were safe at concentrations up to 0.025%. Only loaded and unloaded *NE PG* were non-toxic over the entire range of tested concentrations. LMWH did not show any toxicity at all but HIP led to cell death at a higher concentration. Quaternary ammonium compounds are known for their toxic potential [6] which explains the toxicity at higher concentrations. Additionally, cationic charged lipid-based nanocarriers can interact with anionic membrane proteins, support the binding of the nanocarrier and cause depolarization of the membrane [11]. As a result, also the HIP-loaded formulations showed higher toxicity than the unloaded formulations. In accordance to the hemolysis, NE consisting of polyglycerol-surfactants were less toxic than formulations with PEGylated surfactants as found by Shahzadi et al. [57]. Therefore, polyglycerol-surfactants can be considered as generally safe. The highly toxic potential of *NE PEG PG II* was due to the presence of octanol. Although lysophosphatidylcholine was surface active and cytotoxic to specific cell lines, toxicity towards Caco-2 cells seemed to be low [58]. Consequently, zwitterionic NE were less toxic than NE with PEGylated surfactants but the safety profile was still favourable for NE with polyglycerol-surfactants.

3.7. In vivo study

Up till now, LMWH still has to be administered intravenously or subcutaneously because if its high molecular weight of approximately 5 kDa. Moreover, composition of anionic glycosaminoglycans and hydrophilicity impede absorption in the intestine after oral application [3,16–18]. Another study has already proven that oral application of aqueous LMWH solution led to no noteworthy reduction in Xa activity [19]. Therefore, hydrophilic LMWH was used as a model drug to investigate the influence of different NE on oral bioavailability. Incorporation of LMWH into lipid-based nanocarriers could improve oral bioavailability by taking advantage of their permeation-enhancing effects.

Blood plasma profiles for LMWH are presented in Fig. 7 and main parameters of the study are summarised in Table 3. *NE Z2* was easily digestible and as a result no significant concentration of LMWH was detectable in the plasma. After digestion of lipids or surfactants and destabilization of the droplets, the drug was released and thus could not be absorbed since permeation-enhancing effects of the formulation were lost or decomplexation occurred [6,48]. On the other hand, oral bioavailability around 0.8% and 2.6% were obtained with the non-digestible formulations *NE Z1* and *NE PEG PG II*. Since no lipolysis took place, the HIP remained in the droplets and could benefit from its absorption-promoting effect. Accordingly, non-digestible formulations seem to be favourable to increase oral bioavailability of LMWH. Although LMWH concentration was higher in *NE Z1*, higher plasma concentration was found for *NE PEG PG II*. After dilution with gastrointestinal fluids, more of the hydrophilic complex could have been released from the nanoemulsion and thus was not available for absorption.

4. Conclusion

This study investigated several NE for their influence on oral drug delivery of LMWH. The complex between hydrophilic LMWH and the cationic surfactant didecyldimethylammonium bromide successfully increased lipophilicity and enabled incorporation into NE. Hemolysis assay and the toxicity study provided favourable safety profiles for NE containing polyglycerol-surfactants. Zwitterionic NE exhibited high membrane interaction and the zwitterionic NE without glycerides showed no lipolysis. NE containing polyglycerol-surfactants were more degradable than NE with PEGylated surfactants. It was proven that non-

digestible NE improved oral bioavailability of LMWH. Therefore, non-degradable formulations are promising tools for improving oral drug delivery via lipid-based nanocarriers.

CRediT authorship contribution statement

Katrin Zöller: Conceptualization, Methodology, Investigation, Writing - Original draft, Visualization, Writing - Review & Editing.
Flavia Laffleur: Methodology, Investigation.
Victor Claus: Methodology, Investigation.
Patrick Knoll: Methodology, Investigation, Visualization.
Dennis To: Methodology, Investigation.
Andreas Bernkop-Schnürch: Conceptualization, Writing - Review & Editing, Funding Acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2023.104686.

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