In vivo evaluation of an oral self-emulsifying drug delivery system (SEDDS) for exenatide

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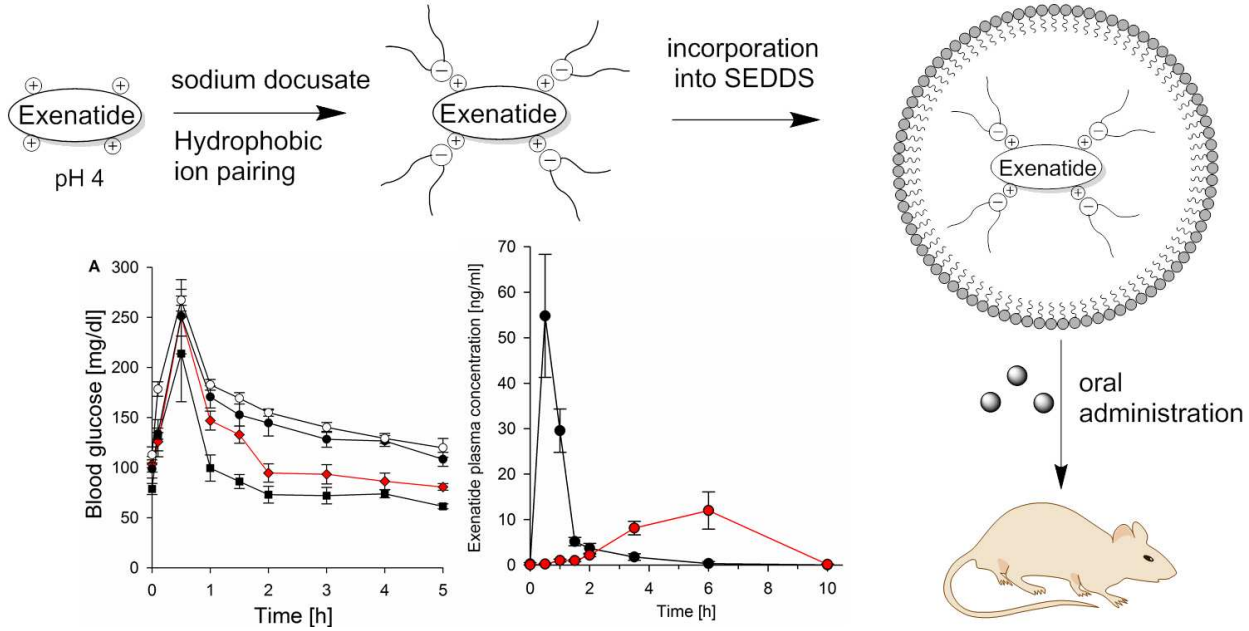
Graphical Abstract



Abstract

Background: The aim of the study was to develop an oral self-emulsifying drug delivery system (SEDDS) for exenatide and to evaluate its *in vivo* efficacy.

Methods: Exenatide was lipidised via hydrophobic ion pairing with sodium docusate (DOC) and incorporated in SEDDS consisting of 35 % Cremophor EL, 25 % Labrafil 1944, 30 % Capmul-PG 8 and 10 % propylene glycol. Exenatide/DOC was characterized in terms of lipophilicity evaluating the octanol/water phase distribution (logP). Exenatide/DOC SEDDS were characterized via droplet size analysis, drug release characteristics (log $D_{SEDDS/release\ medium}$ determination) and mucus permeation studies. Furthermore, the impact of orally administered exenatide/DOC SEDDS on blood glucose level was investigated *in vivo* on healthy male Sprague-Dawley rats.

Results: Hydrophobic ion pairing in a molar ratio of 1:4 (exenatide:DOC) increased the effective logP of exenatide from -1.1 to 2.1. SEDDS with a payload of 1% exenatide/DOC had a mean droplet size of 45.87 ± 2.9 nm and a Log $D_{SEDDS/release\ medium}$ of 1.9 ±0.05. Permeation experiments revealed 2.7-fold improved mucus diffusion for exenatide/DOC SEDDS compared to exenatide in solution. Orally administered exenatide/DOC SEDDS showed a relative bioavailability (versus s.c.) of 14.62 % ± 3.07 % and caused a significant ($p < 0.05$) 20.6 % decrease in AUC values of blood glucose levels.

Conclusion: According to these results, hydrophobic ion pairing in combination with SEDDS represents a promising tool for oral peptide delivery.

Introduction

During the last decades, rates of diabetes mellitus type 2 (T2D) have increased tremendously making it one of the most common causes of death in the world, especially in countries with a high percentage of obesity [1] [2]. Many approaches have been applied in the treatment of this metabolic disorder that is characterized by elevated blood sugar levels, insufficient insulin secretion and/or insulin resistance. One of the more recent developments was the launch of exenatide (marketed as Byetta, Bydureon) in 2005 by Eli Lilly and Company. This 39-amino acid, ~~glucogon-like peptide-1~~ **glucagon-like peptide-1** agonist increases insulin secretion, suppresses pancreatic glucagon release and slows down gastric emptying [3]. As a peptide drug it was developed to be administered by subcutaneous (s.c.) injection. However, adherence to medication is critical for T2D patients, and injectable options are recognised to deter patient compliance [4]. The long-acting injectables also fail to fully recapitulate the temporal nature of the endogenous hormones released from intestinal L-cells during feeding and also the localized first-pass hepatic actions. Injectables have a greater risk of hypo-glycaemia and side-effects such as nausea. Consequently, there is a huge demand for oral delivery systems.

One promising approach to improve oral bioavailability of such peptide drugs is the development of self-emulsifying drug delivery systems (SEDDS) [5]. Belonging to the group of lipid-based formulations, SEDDS can help overcome the main reasons for low oral absorption of peptide drugs, namely enzymatic degradation by intestinal proteases, poor mucus permeating properties and low cellular uptake. For example, it has been already proven, that hydrolyzing enzymes such as pepsin, trypsin, chymotrypsin and elastase are not soluble in the oily SEDDS droplets and that therapeutic peptides incorporated in SEDDS are therefore protected against enzymatic degradation [6]. Further,~~,~~ SEDDS bear a slippery surface facilitating mucus permeation even for

large molecules [7]. Moreover, the absorption membrane permeating properties of SEDDS have been shown in various studies [8].

So far, however, this promising technology has not been utilized for oral delivery of exenatide. **In former studies, oral carrier systems for exenatide included nanoparticles, micelles and microspheres showing promising results and indicating that the use of SEDDS could provide a valuable contribution to the topic [9-12].** It was therefore the aim of this study to increase oral bioavailability of exenatide by incorporating the drug in a SEDDS formulation. A sufficient degree of lipophilicity of a peptide drug is crucial for successful incorporation into the oily droplet core, as such the strategy of hydrophobic ion pairing based on ionic interactions between the peptide and oppositely charged surfactants was utilized [13]. Subsequently, the most promising ion pair was incorporated in a SEDDS formulation that had already shown promise to enhance permeation and provide stability against hydrolysis by lipase [14]. The resulting drug delivery system was investigated, *inter alia*, in terms of drug release, mucus permeating properties and safety. Furthermore, the efficacy of this novel delivery system was evaluated in rats *in vivo*.

Materials and Methods

Materials

Exenatide was purchased from Carbosynth Limited (United Kingdom). Capmul PG-8 EP/NF (propylene glycol monocaprylate, HLB = 6.7) was a gift from Abitec (USA) and Labrafil M 1944 CS was donated by Gattefosse (France). Cremophor EL (polyethoxylated-35 castor oil, HLB = 13), propylene glycol, sodium docusate, sodium dodecylsulfate, sodium oleate, sodium

taurocholate and all other chemicals, reagents and solvents were purchased from Sigma Aldrich (Germany).

Quantification of exenatide

The quantification of exenatide was performed via HPLC. In brief, samples containing exenatide were analyzed on a Nucleosil 100-5 C18 column (250 × 4 mm) at 40 °C with gradient elution (0.5 mL/min): 0–20 min; linear gradient; from 58% A/42% B to 26% A/74% B (eluent A: 0.1 % trifluoracetic aicid in water; eluent B: 0.1% trifluoroacetic acidin 80% acetonitrile) with signal dtermination by UV/VIS at 278 nm. Retention time was 8.7 min. The calibration curve was established with exenatide in a range from 7.8 to 1000 µg/mL. The detection and quantification limit was investigated in a validation study and was specified with 2 µg/mL and 3.9 µg/mL.

Hydrophobic ion pairing of exenatide

The strategy of hydrophobic ion pairing was applied to increase lipophilicity of exenatide and facilitate incorporation into SEDDS. In order to identify the most promising candidate, different surfactants were tested. Exenatide acetate was dissolved in 0.555 mM acetic acid (pH 4) to a final concentration of 2 mg/mL. Sodium dodecylsulfate, sodium deoxycholate, sodium docusate, sodium oleate and sodium taurocholate were dissolved in 0.555 mM acetic acid (pH 4) and added dropwise to the exenatide solution under light shaking in a ratio of 1:1 (v/v). Surfactant solutions were prepared in different concentrations in order to obtain molar ratios of 1:1, 1:2, 1:4 and 1:6 (exenatide:surfactant). The precipitated ion-pair was separated by centrifugation at 13000 rpm (MiniSpin®, Eppendorf Austria GmbH). The supernatant was analyzed for remaining un-precipitated exenatide via HLPC as described above. The ion pair was washed with water, lyophilized at −30 °C and 0.01 mbar (Christ Gamma 1-16 LSC Freeze dryer) and stored at −24 °C.

Determination of LogP (octanol/water) of exenatide/DOC and exenatide/SDS ion pairs

Ion pairs of exenatide with SDS and DOC in molar ratios of 1:1, 1:4 and 1:6 were prepared as described above and the pH adjusted to 6.8 with NaOH. Afterwards, octanol was added to the mixture in a volume ratio of 1:1 (octanol:aqueous phase). The mixtures were placed on a thermomixer (900 rpm, 25 °C) for 30 min followed by phase separation via centrifugation (5 min, 13000 rpm, MiniSpin®, Eppendorf Austria GmbH)). The amount of exenatide in octanol and water phase was determined via HPLC as described above. Octanol samples (10 µL) were diluted prior to injection in HPLC with isopropanol (80 µL) and water (110 µL) to yield a final volume of 200 µL. The same experiment was performed with aqueous exenatide solution that was adjusted to pH 6.8 with NaOH. Furthermore, 100 % values were generated in order to exclude the presence of exenatide in the interphase.

Preparation and characterization of SEDDS

For SEDDS development, the ion-pair exenatide/DOC in a ratio of 1:4 has been chosen. A previously developed SEDDS formulation showing protection against enzymatic degradation was employed. The proportions of the individual components were slightly adapted in order to identify the most suitable composition for the incorporation of the ion pair. Therefore, different ratios of Cremophor EL, Labrafil 1944, Capmul PG-8 and propylene glycol were homogenized by vigorous stirring. The lyophilized exenatide/DOC was dissolved in a concentration of 1 % in the pre-concentrate for *in vitro* studies. This concentration was found to be the maximum soluble payload in the SEDDS formulation referring to an exenatide payload of 0.71 %. For *in vivo* studies the ion-pair was dissolved in a concentration of 0.0842 % in order to reach an applicable volume of 250 µL. For *in vitro* studies SEDDS were emulsified in 50 mM phosphate buffer pH 6.8 in a concentration of 20 % (m/v) under gentle shaking. The time of emulsification was

evaluated visually. The mean droplet size and the zeta potential were measured by dynamic light scattering using a PSS NICOMP TM 380 DLS (Santa Barbara, CA, USA). SEDDS without incorporated exenatide served as control.

Determination of log D $_{\text{SEDDS/release medium}}$ of exenatide/DOC

Log D $_{\text{SEDDS/release medium}}$ was calculated as a measure of the distribution of exenatide/DOC between the oily droplet phase of SEDDS and the release medium. Therefore, lyophilized exenatide/DOC (molar ratio 1:4) was dispersed in water, 50 mM phosphate buffer pH 6.8 or 100 mM HCl pH 1 in a concentration of 1 mg/mL. The suspensions were stirred for 6 hours and centrifuged (13000 rpm, MiniSpin®, Eppendorf Austria GmbH). The supernatant was analyzed by HPLC with respect to solubility of the ion pair in the respective release medium (C_{RM}). The solubility of the ion pair in the SEDDS concentrate corresponds to the maximum payload of 1 % (C_{SEDDS}). D values were calculated according to equation 1. The drug concentration in SEDDS depending on the ratio between SEDDS (V_{SEDDS}) and aqueous release medium (V_{RM}) was calculated according to equation 2.

$$\log D = \log \frac{C_{RM}}{C_{SEDDS}} \qquad \textit{(equation 1)}$$

$$C_{SEDDS}\,(\%) = \frac{100\,\%}{1 + \frac{V_{RM}}{V_{SEDDS} * D}} \qquad \textit{(equation 2)}$$

Release studies

Release studies were performed via dialysis membrane method. Exenatide/DOC containing SEDDS pre-concentrate was emulsified in 50 mM phosphate buffer pH 6.8 (20 %, m/v), and 1.75 mL of the resulting emulsion was further diluted with phosphate buffer to a total volume of 2.5 mL **in order to approach sink conditions and to minimize the membrane effect** leading to a

final exenatide concentration of 1 mg/mL. The same volume and concentration of exenatide acetate in 50 mM phosphate buffer pH 6.8 served as control. Test solutions were filled into dialysis tubes and placed in beakers with 10 mL 50 mM phosphate buffer pH 6.8. The release of exenatide to the outer phase beyond the confines of the dialysis tube was analyzed via HPLC as described above over 6 h.

Mucus diffusion studies

Investigation of mucus permeating properties of exenatide/DOC SEDDS were performed with transwell® with ThinCerts (surface 33.6 mm^2, pore size 0.4 µm, pore density (Greiner-BioOne, Austria)) in 24-well plates and an amount of 60 mg of mucus [15]. Briefly, 250 µL of exenatide/DOC SEDDS (20 % in 50 mM phosphate buffer pH 6.8) and the corresponding amount of exenatide in 50 mM phosphate buffer pH 6.8 (1.426 mg/mL) were layered carefully over the mucus in the donor compartment. The acceptor chamber was filled with 500 µL 50 mM phosphate buffer pH 6.8. The plate was incubated at 37 °C under continuous shaking at 20 rpm on an orbital shaker. At predetermined time points 100 µL aliquotes were withdrawn from the acceptor compartment and replaced with the same volume of fresh buffer. **The experiment was performed over a time interval of 4 hours corresponding to the time of a complete mucus turnover in the small intestine [16, 17].** The amount of permeated exenatide was calculated via HPLC as described above with reference to a 100% control that was obtained by the same procedure but without mucus in the donor chamber.

Resazurin assay

In order to examine cytotoxic potential of exenatide/DOC SEDDS a resazurin assay on Caco-2 cells was performed [18]. Briefly, Caco-2 cells were seeded on a 24 well plate (d = 1 × 105 cells/well; 500 µL per well) in minimum essential medium (MEM) supplemented with 10% (v/v)

fetal calf serum (FCS) and penicillin/streptomycin solution (100 units/0.1 mg/L) and cultured for 2 weeks at 37 °C in an atmosphere of 5% CO^2 and 95% relative humidity. During this period the medium was changed every other day. Subsequently, cells were washed with phosphate buffered saline (PBS) with a temperature of 37°C and treated with 500 µL of test solutions **of different concentrations from 2.5 mg/mL to 7 mg/mL**. Pure MEM served as negative control and 1% (w/v) Triton X 100 as positive control. After incubation time of 12 h cells were washed with PBS and treated with a 5% (m/v) resazurin solution. Cells were incubated for additional 2 h and fluorescence of the supernatant was measured at 540 nm excitation wavelength and 590 nm emission wavelength (TECAN Infinite M200, Austria GmbH).

In vivo studies

In vivo studies were approved by the Ethical Committee of Austria and performed according to the Principles of Laboratory Animal Care. Male Sprague-Dawley rats with a mean body weight of 250–300 g were obtained from Janvier Labs (Saint Berthevin, France). For pharmacokinetic studies, rats were randomly devided into 2 groups (n=3). The first group served as positive control and received 60 µL of s.c. exenatide injection (0.333 mg/mL in PBS). The second group received 150 µg of exenatide via oral gavage in form of 250 µL SEDDS pre-concentrate with exenatide/DOC (0.0842 %, m/m). Blood samples were collected at time intervals of 0, 0.5, 1, 1.5, 2, 3.5, 6 and 10 hours following exenatide administration. Exenatide was extracted from plasma according to a protocol from Phoenix Pharmaceuticals Inc.. In brief, samples were acidified by equal volume of acidic buffer and centrifuged for 20 minutes at 17,000 x g (4°C). The collected supernatant was loaded into a C18 column that was pretreated with the same buffer. Exenatide was eluted from the column utilizing a second buffer supplied by Phoenix Pharmaceuticals Inc.. Then, exenatide buffer solution was freeze dried and exenatide concentration was measured by Chemiluminescent Elisa technique (Phoenix Pharmaceuticals Inc). The concentration-time curve

was plotted and AUCs were measured by trapezoidal rule for all exenatide systems. Relative bioavailability was evaluated according to equation 3.

$$Relative\ bioavailability\ [\%] = \left(\frac{[AUC]oral * [Dose]S.C}{[AUC]S.C * [Dose]oral}\right) * 100 \qquad \text{(equation 3)}$$

For pharmacodynamics studies, rats were randomly divided into 4 groups (n = 3). The first group served as positive control and received 60 µL of s.c. exenatide injection (0.333 mg/mL in PBS) and 10 min later 2 g/kg glucose by i.p. route (50% dextrose solution in sterile water). The second and third group received 150 µg of exenatide via oral gavage followed by 2 g/kg glucose i.p. 3 hours later. The second group received the drug in form of 250 µL exenatide solution (0.6 mg/mL in PBS) and the third group in form of 250 µL SEDDS pre-concentrate with exenatide/DOC (0.0842 %, m/m). The fourth group served as negative control and received only 2 g/kg glucose via i.p. route. The animals were fasted 12 h prior to oral administration and had free access to water during the experiment. At predetermined time points blood samples were taken from the tail vein and blood glucose was measured immediately by *Glucometer AccuCheck* ® Active (F. Hoffmann-La Roche AG).

Statistical data analysis

The software Sigma Plot version 12.3 was used for the statistical data analysis. One way ANOVA and Bonferroni t-test were performed with $p<0.05$ as the minimal level of significance.

Results and discussion

Preparation and characterization of ion-pairs

The strategy of hydrophobic ion-pairing was used in the present study to increase lipophilicity and hence solubility of exenatide in the SEDDS pre-concentrate. Since complex formation via ion pairing is based on ionic interactions without any chemical modification the peptide functionality is maintained. In this study different surfactants in different ratios were tested. Oleic acid, sodium deoxycholate and sodium taurocholate did not lead to any ion pair formation indicating that obviously only surfactants with a permanent and pH-independent negative charge precipitate with exenatide in acid environment. Therefore, sodium dodecyl sulfate and sodium docusate were added in different ratios to exenatide solutions in 0.555 mM acetic acid pH 4. Upon addition of surfactant to the peptide solution an immediate precipitation of ion pair was observed. The extent of ion pair formation was evaluated by quantifying un-precipitated peptide in the supernatant via HPLC. Figure 1 shows the percentage of precipitated exenatide depending on the amount of added surfactant.

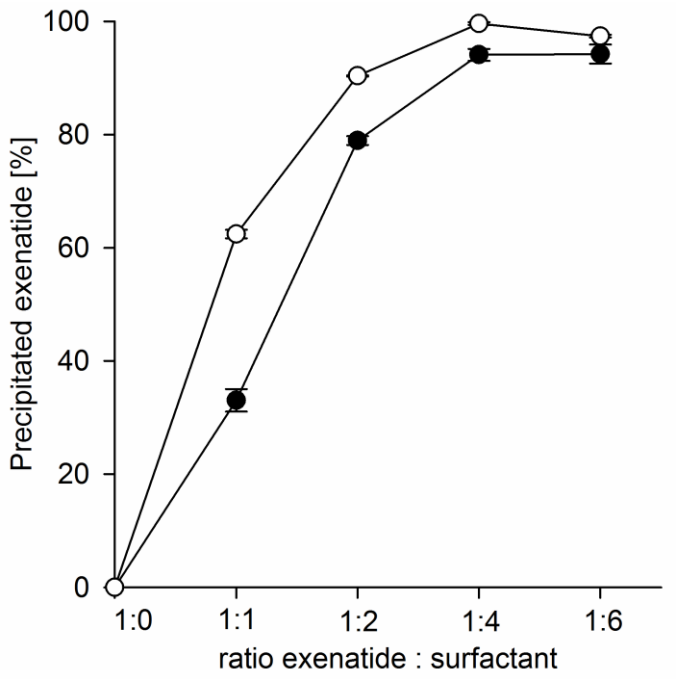


Figure 1: ~~Precipitation of exenatide from 0.555 mM acetic acid (2 mg/mL) with DOC (●) and SDS (○).~~ Precipitation of exenatide from 0.555 mM acetic acid (2 mg/mL) with DOC (○) and SDS (●). The precipitated exenatide-surfactant ion pair was isolated by centrifugation and the remaining exenatide analysed by HPLC. Data are shown as mean ± SD (n = 3).

In a molar ratio of 1:4 (surfactant:exenatide) both surfactants reached the maximum of precipitated ion pair. This result is in accordance with the structure of exenatide: At pH 4, four positive net charges can be assumed at position 1, 12, 20 and 27 in the amino acid sequence that can form ionic complexes with four molecules of negatively charged surfactant. For each tested ratio, DOC showed a higher rate of ion pair formation indicating superiority over SDS as surfactant. This was also confirmed by the partitioning coefficient (logP) values of exenatide/SDS and exenatide/DOC in different ratios (Figure 2). Determining the octanol/ water partitioning coefficient of ion pairs provides information about their lipophilicity that is crucial for efficient incorporation in SEDDS. The control experiment with exenatide alone proved the hydrophilic nature of the peptide with a log P value of -1.14. With increasing amounts of surfactant, log P values rapidly increases to 1.6 (SDS) or 2.1 (DOC). Both surfactants increased

lipophilicity most at a ratio of 1:4. At a ratio of 1:6 (surfactant:exenatide) log p values slightly decreased. This might be due to formation of micellar complexes diffusing into the water phase. According to these results, docusate in a molar ratio of 1:4 (exenatide:DOC) was chosen for further experiments.

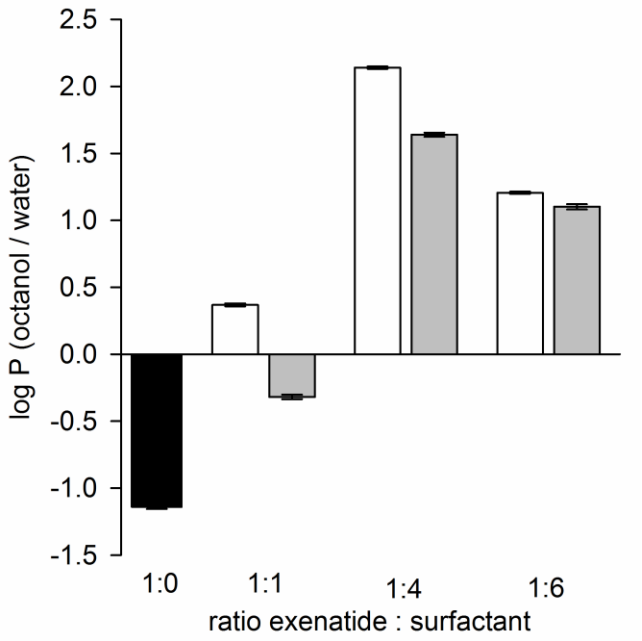


Figure 2: Log P (octanol/water) of aqueous exenatide solution pH 6.8 (black bar) and of exenatide/DOC (white bars) and exenatide/SDS (grey bars) at pH 6.8 depending on ratios between peptide and surfactant. Values are means of at least three experiments ± SD.

SEDDS development and characterization

Most of orally administered SEDDS formulations are prone to hydrolysis by pancreatic lipase, an enzyme naturally occurring in duodenum, cleaving triglycerides in positions 1 and 3, resulting in two free fatty acids and one 2- monoacylglyceride [19] [14]. The resulting deconstruction of the emulsion can lead to enzymatic degradation of the ion pair under physiological conditions and an unsatisfying *in vitro/ in vivo* correlation. In order to improve the *in vivo* situation, only lipolysis stable components have been chosen in this study. The main oily components of the final SEDDS concentrate are Labrafil 1944 and Capmul PG-8, both lacking classical triglyceride structure being therefore stable against digestion. Furthermore, all components proved to be stable against hydrolysis by lipase as confirmed in a former study [14]. The formation of stable SEDDS in the gastro-intestinal tract needs a surfactant concentration ranging from 30-60 % (m/m). Cremophor-EL was chosen because of its high HLB value ensuring high self-emulsifying properties and small droplet sizes [20] and being less toxic compared to other ionic surfactants [21]. For developing the SEDDS pre-concentrate, different ratios of surfactant and oily components were tested. Each formulation contained propylene glycol as a co-solvent in a concentration of 10%. According to a former study, the amount of gastrointestinal fluid in fasted rats is 3.2 ± 1.8 mL [22]. In this study the amount of applied SEDDS formulation to the animals was 250 µL corresponding to a SEDDS concentration of 7.8 % (m/v). However, as the SEDDS formulation is not emulsified in the whole gastrointestinal fluid, a concentration of 20 % (m/v) in aqueous phase was applied to test *in vitro* the emulsifying properties. Finding the most suitable composition for the pre-concentrate, the focus was set on fast emulsification and a small droplet size with a low PDI value. Table 1 shows the pre-concentrate with the most favorable self-emulsifying properties with and without incorporated ion pair. This composition was used for any further experiments. The increase in mean droplet size due to addition of the ion pair previous to emulsification

indicates successful incorporation into the SEDDS droplets. Due to incorporation of the ion pair into SEDDS, no precipitation was observed. Zeta potential measurements revealed an almost uncharged surface for blank SEDDS and exenatide/DOC SEDDS.

Table 1: ~~Composition, droplet size and polydispersity index of blank and loaded SEDDS in a dilution of 20 % (m/v) in 50 mM phosphate buffer pH 6.8.~~ **Composition, droplet size, polydispersity index and zeta potential of blank and loaded SEDDS in a dilution of 20 % (m/v) in 50 mM phosphate buffer pH 6.8. Parameters were measured by dynamic light scattering using a PSS NICOMP TM 380 DLS (Santa Barbara, CA, USA).** Values are means of at least three experiments ± SD.

formulation	components	amount [%]	mean droplet size [nm]	PDI	zeta potential [mV]	emulsification time	appearance
SEDDS concentrate	Cremophor EL	35	30.4 ± 4.5	0.128	-0,1 ± 0.7	< 1 min	slightly bluish
	Labrafil 1944	25					
	Capmul PG-8	30					
	Propylene glycol	10					
SEDDS + Exenatide	SEDDS concentrate	99	45.87 ± 2.9	0.151	-0.7 ± 0.1	< 1 min	slightly bluish
	Exenatide	1					

Release studies

Release studies show the time dependent release of the drug from the oily SEDDS droplets into the release medium. For quantification of the drug released from SEDDS the oily droplets need to be separated. This was achieved using a separating dialysis membrane. The release profile of exenatide/DOC SEDDS is displayed in Figure 3. As can be seen in the graph, this method has a tremendous impact on the resulting release profile. Currently there is no valid method to circumvent this problem determining the drug release from SEDDS *in vitro* to a satisfying extent. In order to study this membrane effect and its influence on the release profile, the increase in

drug concentration in the acceptor medium was quantified with exenatide in the donor compartment without any formulation. The time dependent increase in quantified drug from the acceptor medium shows clearly the influence of the membrane. Taking this effect into account, the release profile from the SEDDS formulation does not show any sustained release and is simply based on the effect of the distribution of the ion pair between the oily phase and the aqueous phase resulting in a lower drug concentration in the aqueous donor medium and consequently in a flatter concentration gradient. As no sustained release could be observed, the presence of fluid-crystalline partial structures in the SEDDS droplets is very unlikely, which was confirmed by polarized microscopic analysis.

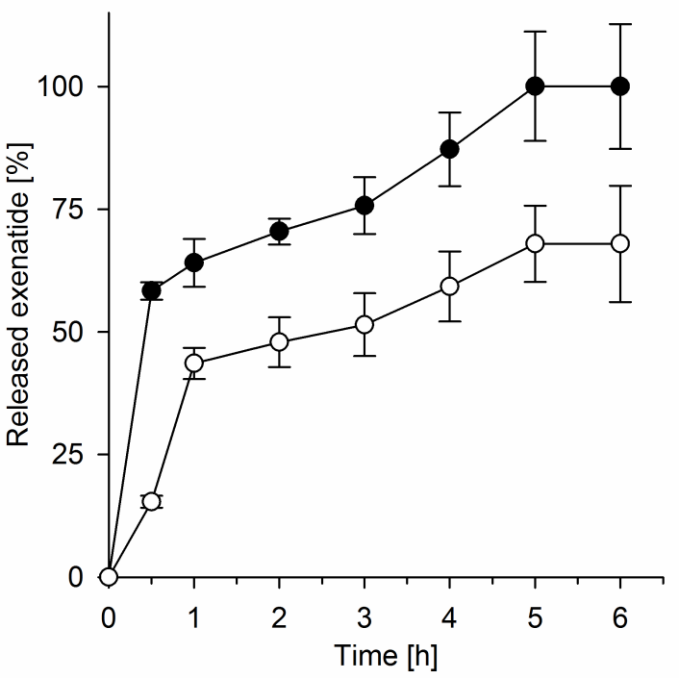


Figure 3: In vitro release of exenatide from solution (50 mM phosphate buffer pH 6.8) [●] and from exenatide/DOC SEDDS [○] at 37 °C over 6 h. Values are means of at least three experiments ± SD.

This data interpretation is confirmed by latest observations in this field [23]. The authors postulate in this article that drug release from SEDDS is based on a simple diffusion process from the oily droplet phase into an aqueous phase taking place within seconds. The drug release is thereby just controlled by the partition coefficient (log D) of the ion pair between the lipophilic SEDDS phase and the release medium. In the release study this phase distribution is set up immediately after each sampling. Table 2 shows the log $D_{\text{SEDDS/release medium}}$ values dependent on different release media. In the experimental setup, phosphate buffer was used as release medium and the ratio between SEDDS and release medium was 1:35. The phase distribution between SEDDS and buffer should be 70 % vs 30 %. Taken the membrane effect into account, the immediate release of 20 % exenatide after 30 min is confirming this assumption.

Table 2: Log D (SEDDS/release medium) of exenatide/DOC (1:4). Values are means of at least three experiments ± SD.

Release medium	Log D
Water	3.0 ± 0.01
100 mM HCl pH 1	3.2 ± 0.03
50 mM phosphate buffer pH 6.8	1.9 ± 0.05

Predicting the *in vivo* situation, a total volume of gastrointestinal fluid in rats of 3.2 mL (plus 0.25 mL water given by oral gavage to wash down SEDDS formulation) was assumed, meaning that 85.2 % of the ion pair remain in the SEDDS droplets and only 14.8 % are released immediately probably not reaching the epithelium.

Mucus diffusion studies

One of the main reasons for poor drug absorption of peptide drugs is the mucus gel layer that is composed of negatively charged glycoproteins covering the gastro intestinal tract. In order to

reach the epithelium drugs must overcome this natural barrier. Many substances and especially peptide drugs get entrapped in this layer by ionic interactions with the glycoproteins. SEDDS showed in various studies the potential to overcome this barrier due to their lipophilic nature, no or negative surface charge and small droplet size [7, 24, 25]. Results from permeation studies are provided in Figure 4 and show 2.7-fold improved mucus permeation for exenatide containing SEDDS compared to the drug without formulation after four hours. The permeation enhancing properties of Cremophor-EL itself might also contribute to this result. This surfactant showed also the ability to open tight junctions of cells improving drug uptake [26]. However, the exact mechanism of drug uptake from and/or of SEDDS has so far not been thoroughly understood. Possible mechanisms are drug release outside the cell followed by uptake of the drug alone, fusion of droplets with the bilayer of the cell membrane, passage of SEDDS through the membrane by opening of tight junctions or the uptake of the entire SEDDS droplet via endocytosis or even transcytosis.

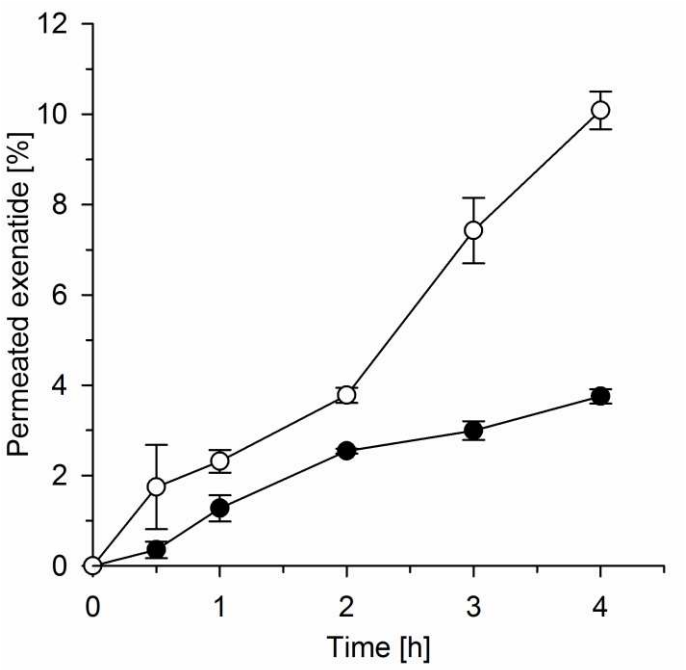


Figure 4: Mucus permeation study using transwell® system over 4 h at 37 °C. Percentage of permeated exenatide as a function of time: exenatide solution (14.3 mg/mL in 50 mM phosphate buffer pH 6.8) (●) and exenatide/DOC SEDDS (○). Values are means of at least three experiments ± SD.

Resazurin assay

Drug delivery systems that diffuse through the mucus layer bear the risk of cell damaging effects on the underlying membrane. Basically all so far published SEDDS show cell-toxic effects *in vitro* due to their high concentration of surfactants being able to penetrate and destroy the cell membrane. However, *in vitro* tests might not state actual *in vivo* conditions. Physiological sink conditions reduce concentration and toxic effects over time. Furthermore, unlike cells of the intestinal epithelium, Caco-2 cells are not protected by a mucus layer. Even enzyme containing and highly mucus penetrating nanocarriers do not destroy this protective barrier [27]. According to already published data, the liquid volume in the small intestine of humans ranges from 30 to 420 mL in fasted state and from 18 to 660 mL in the fed state [28]. Considering that SEDDS are diluted to compensable concentrations under physiological conditions and that the mean small

intestinal passage time is 4 hours, a maximum concentration of 5 mg/mL (m/v) and an incubation time of 4 hours was chosen in former studies [14]. In the present study, parameters were adjusted to a maximum concentration of 7 mg/mL and an incubation time of 12 hours ~~in order to cover~~ covering a larger range of concentration and dwell time ~~in the body~~ **in order to study the full cytotoxic potential over a longer time period in case of accumulation in the body**. Figure 5 shows the influence of exenatide/DOC SEDDS on cell viability depending on SEDDS concentration. A positive correlation between SEDDS concentration and cell toxicity could be observed. At a concentration of 5 mg/mL cell viability was still at 70 %. This result is in good agreement with former published results [14]. In the mentioned study, a blank SEDDS formulation containing the same compounds as exenatide/DOC SEDDS showed a cell viability of 94.2 % after 4 hours. Considering the 3-fold longer incubation time in the present study, the 20 % lower survival rate is well explainable. **Results obtained from the cell disrupting substance Triton x proved the reliability of the chosen method as measured fluorescence and hence cell viability was remarkably decreased compared to the 100 % value of MEM treated cells**

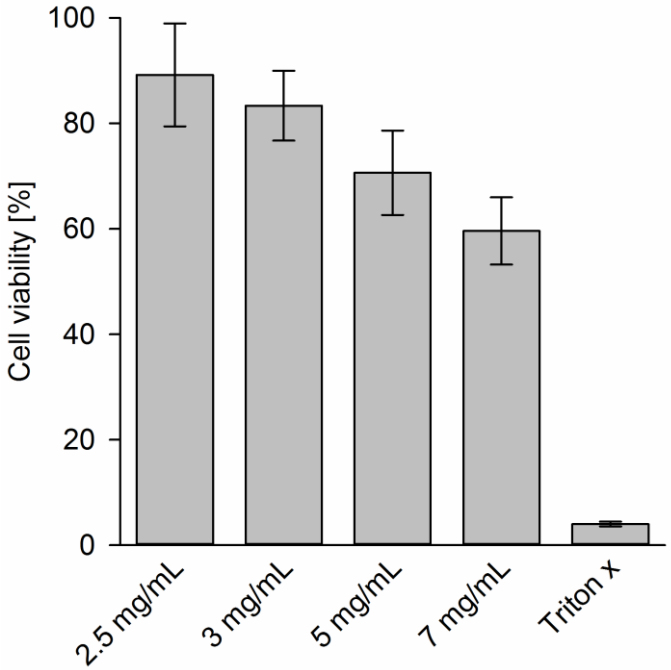


Figure 5: Cytotoxicity of exenatide/DOC SEDDS after 12 h incubation in concentrations of 2.5 mg/mL, 3 mg/mL, 5 mg/mL and **7 mg/mL**~~10 mg/mL~~. Values are means of at least three experiments ± SD.

In vivo study

Results from pharmacokinetic studies are provided in Table 3 revealing a relative bioavailability of exenatide/DOC SEDDS vs s.c. exenatide solution of 14.62 ± 3.07 %. As illustrated in Figure 6, plasma concentrations of exenatide s.c. reached their maximum after 0.5 h, whereas the plasma concentrations of exenatide/DOC SEDDS were at their highest after 6 h due to a sustained absorption from gastrointestinal tract. According to literature, orally applied exenatide solution has a very low bioavailability with no detectable exenatide in plasma [29] [30]. Due to incorporation into SEDDS a relative bioavailability of almost 15 % could be achieved being in the same range [30] or even exceeding [29] results from former studies that used particulate carrier systems for oral exenatide application. According to data from literature, absolute bioavailability of exenatide s.c. compared to i.v. is 61.7 %, hence absolute bioavailability of exenatide/DOC SEDDS vs i.v. application is likely 9.0 %. Accordingly, exenatide/DOC SEDDS

showed a strikingly improved absolute bioavailability compared to already published peptide containing SEDDS showing absolute bioavailability values below 2.5 % (oral vs i.v.) [14] [26].

Table 3: Pharmacokinetic data of the in vivo study **for orally administered exenatide/DOC SEDDS (dose: 150 µg) and s.c. exenatide solution (dose: 20 µg).**

Formulation	C_{max} [ng/mL]	T_{max} [h]	AUC_{0-10} [ng h/mL]	Relative bioavailability [%]	Absolute bioavailability [%]
exenatide s.c.	54.80 ± 13.53	0.5	53.16 ± 3.84	100	61.7
exenatide/DOC SEDDS	~~8.12 ± 1.48~~ **11.99 ± 4.10**	6	58.79 ± 15.83	14.62 ± 3.07	9 ± 1.89

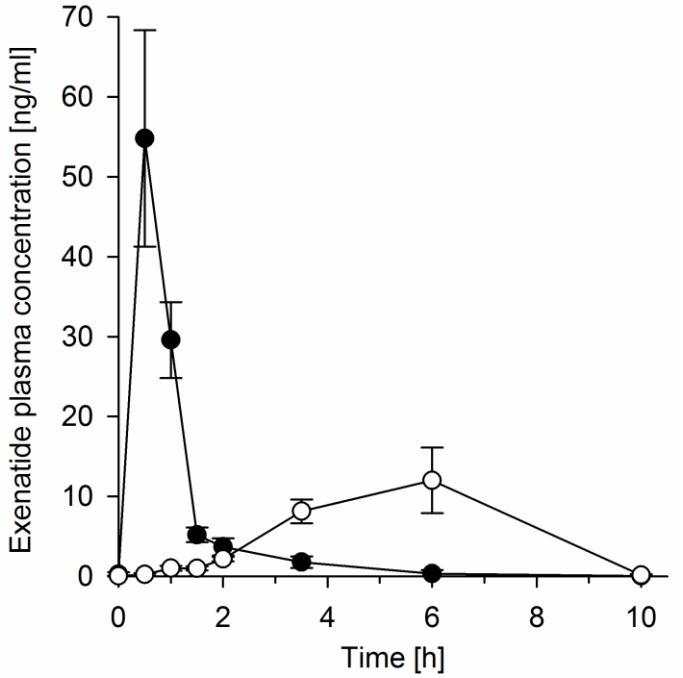


Figure 6: Exenatide plasma concentration-time curve for orally administered exenatide/DOC SEDDS **(dose: 150 µg)** (○) and s.c. exenatide solution **(dose: 20 µg)** (●). Illustrated values are the means of at least three experiments ± standard deviation

Since exenatide shows high activity, pharmacodynamic effects can be measured even at low plasma concentrations. Exenatide binds to the GLP-1 receptor causing a glucose-dependent insulin secretion followed by enhanced insulin response [31]. Therefore, time dependent blood glucose levels were measured after glucose challenge. According to the manufacturer, exenatide should be administered prior to meal times [31]. For s.c injection an interval of 40 min between administration and glucose challenge was chosen and 3 hours for oral administered formulations. The measured blood glucose levels over time are shown in Figure 7 (A). As the experiment was performed with healthy rats, each group showed a fast decrease in blood glucose levels after the initial glucose peak due to sufficient insulin response. However, this insulin response was significantly increased in animals treated by s.c injection and orally administered SEDDS formulation ($p < 0.05$). Thereby, the SEDDS caused a 20 % lower blood glucose level one hour after glucose challenge compared to the control only treated with exenatide solution. After two hours, this effect was most pronounced with 38.9 % lower blood glucose levels in the SEDDS group compared to the control group. Even after 5 hours blood glucose levels in the SEDDS group were lower compared to the control. This might be due to the comparably long half-life of the drug. Exenatide levels can be measured for approximately 10 hours bearing the risk of hypoglycemia [32]. Figure 7 (B) shows the calculated AUC values of plasma glucose levels. In the SEDDS group the AUC was 20.6 % smaller than in the control group. Furthermore, no significant difference between the AUC of orally applied exenatide solution compared to the control was observed ($p < 0.05$). According to these results there is strong evidence that incorporation in SEDDS could enhance oral bioavailability of exenatide. Due to the high potency of this drug, the usual problems of SEDDS formulations, e.g. low payload and low oral drug absorption compared to s.c application, do not matter that much.

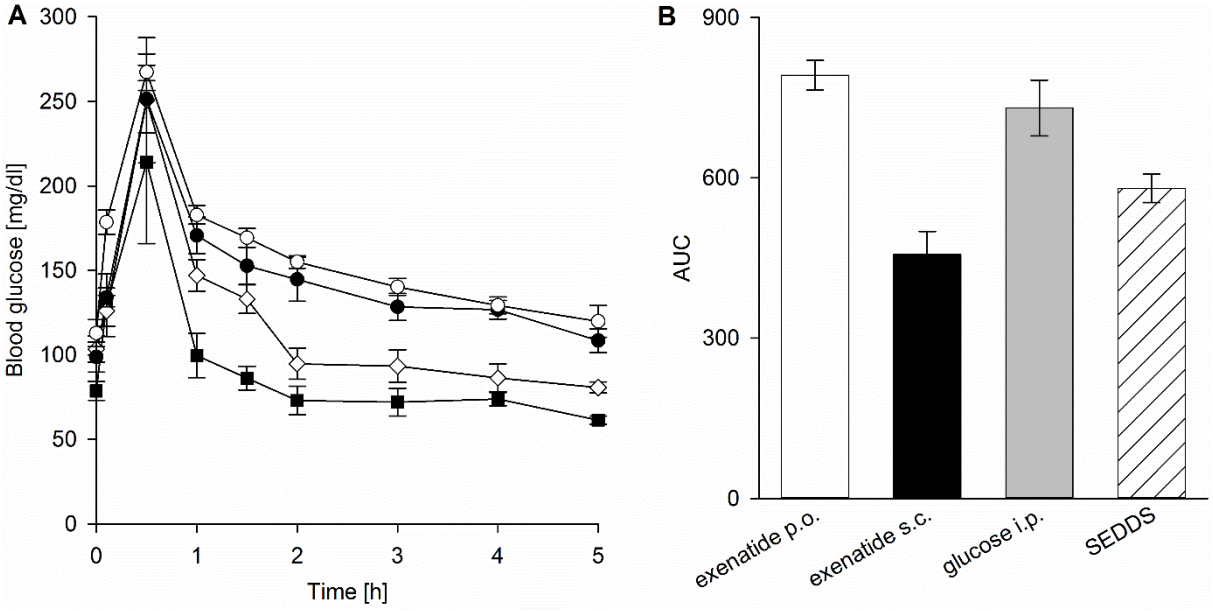


Figure 7 (A+B): In vivo evaluated pharmacodynamics **(A)** Time dependent plasma glucose levels of rats treated with exenatide s.c. + glucose i.p. (■), exenatide/DOC SEDDS p.o. + glucose i.p. (◊), exenatide p.o. + glucose i.p. (○) and glucose i.p. alone (●). **(B)** AUC of plasma glucose levels. Values are means of at least three experiments ± SD.

Conclusion

In the present study a lipid based drug delivery system for oral administration of exenatide has been established. Release studies with exenatide/DOC SEDDS confirmed latest observations in this field of research. The data of the present study could therefore be an important step in the investigation and optimization of peptide containing SEDDS. **Cell viability studies via resazurin assay showed a concentration dependent membrane disrupting effect of exenatide/DOC SEDDS being in good correlation with all so far published data.**

Considering *in vivo* situation in the human body, however, SEDDS are way more diluted and cells protected by a mucus barrier providing likely sufficient safety. Mucus diffusion experiments as well as ~~Pharmacokinetic~~ pharmacokinetic and pharmacodynamic studies revealed promising results indicating high *in vitro* / *in vivo* correlation: ***In vitro*** **permeation studies revealed 2.7-fold improved mucus permeation for exenatide containing SEDDS compared to the drug without the formulation. *In vivo* evaluation showed a 20.6 % decreased AUC of plasma glucose in the SEDDS group vs. the control group**. This might be on the one hand due to the high lipophilicity of the created ion pair being incorporated into SEDDS and on the other hand due to the high potency of the peptide, enhancing insulin response even at low plasma concentrations. Considering that only healthy animals were tested in this study, effects on plasma glucose levels might be even more pronounced in type-2 diabetics.

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Table 1: ~~Composition, droplet size and polydispersity index of blank and loaded SEDDS in a dilution of 20 % (m/v) in 50 mM phosphate buffer pH 6.8.~~ **Composition, droplet size, polydispersity index and zeta potential of blank and loaded SEDDS in a dilution of 20 % (m/v) in 50 mM phosphate buffer pH 6.8. Parameters were measured by dynamic light scattering using a PSS NICOMP TM 380 DLS (Santa Barbara, CA, USA).** Values are means of at least three experiments ± SD.

formulation	components	amount [%]	mean droplet size [nm]	PDI	zeta potential [mV]	emulsification time	appearance
SEDDS concentrate	Cremophor EL	35	30.4 ± 4.5	0.128	-0,1 ± 0.7	< 1 min	slightly bluish
	Labrafil 1944	25					
	Capmul PG-8	30					
	Propylene glycol	10					
SEDDS + Exenatide	SEDDS concentrate	99	45.87 ± 2.9	0.151	-0.7 ± 0.1	< 1 min	slightly bluish
	Exenatide	1					

Table 2: Log D (SEDDS/release medium) of exenatide/DOC (1:4). Values are means of at least three experiments ± SD.

Release medium	Log D
Water	3.0 ± 0.01
100 mM HCl pH 1	3.2 ± 0.03
50 mM phosphate buffer pH 6.8	1.9 ± 0.05

Table 3: Pharmacokinetic data of the in vivo study **for orally administered exenatide/DOC SEDDS (dose: 150 µg) and s.c. exenatide solution (dose: 20 µg).**

Formulation	C_{max} [ng/mL]	T_{max} [h]	AUC_{0-10} [ng h/mL]	Relative bioavailability [%]	Absolute bioavailability [%]
exenatide s.c.	54.80 ± 13.53	0.5	53.16 ± 3.84	100	61.7

exenatide/DOC SEDDS	~~8.12 ± 1.48~~ **11.99 ± 4.10**	6	58.79 ± 15.83	14.62 ± 3.07	9 ± 1.89

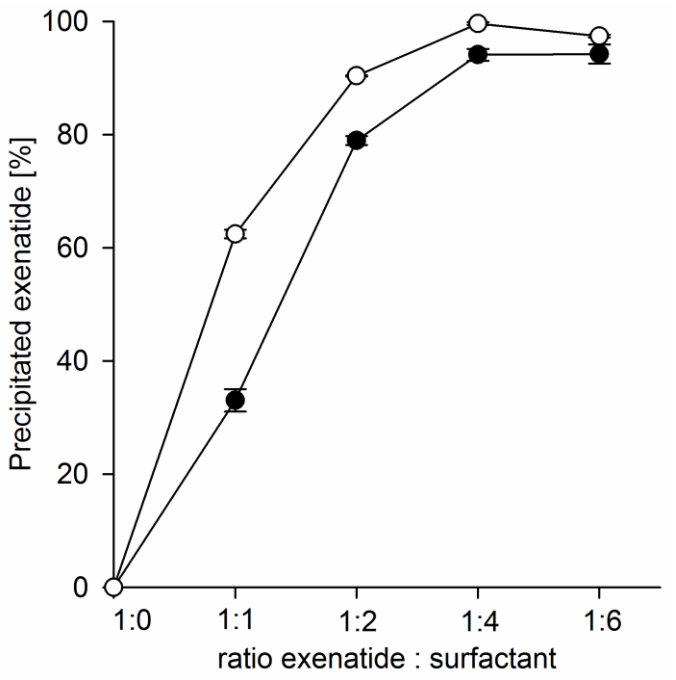

Figure 1: ~~Precipitation of exenatide from 0.555 mM acetic acid (2 mg/mL) with DOC (●) and SDS (○).~~ Precipitation of exenatide from 0.555 mM acetic acid (2 mg/mL) with DOC (○) and SDS (●). The precipitated exenatide-surfactant ion pair was isolated by centrifugation and the remaining exenatide analysed by HPLC. Data are shown as mean ± SD (n = 3).

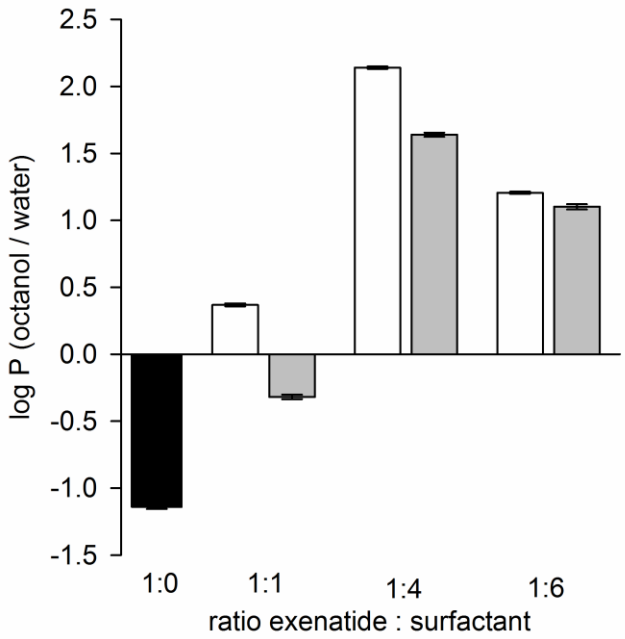

Figure 2: Log P (octanol/water) of aqueous exenatide solution pH 6.8 (black bar) and of exenatide/DOC (white bars) and exenatide/SDS (grey bars) at pH 6.8 depending on ratios between peptide and surfactant. Values are means of at least three experiments ± SD.

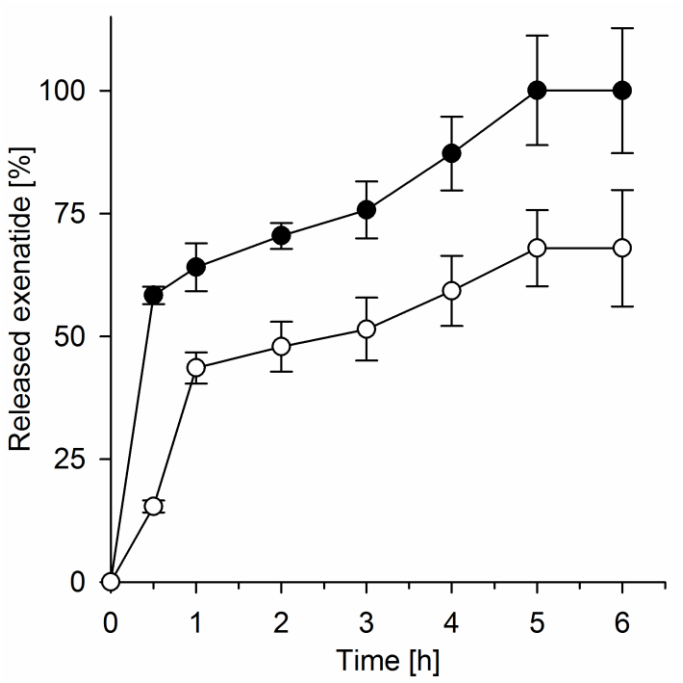

Figure 3: In vitro release of exenatide from solution (50 mM phosphate buffer pH 6.8) [●] and from exenatide/DOC SEDDS [○] at 37 °C over 6 h. Values are means of at least three experiments ± SD.

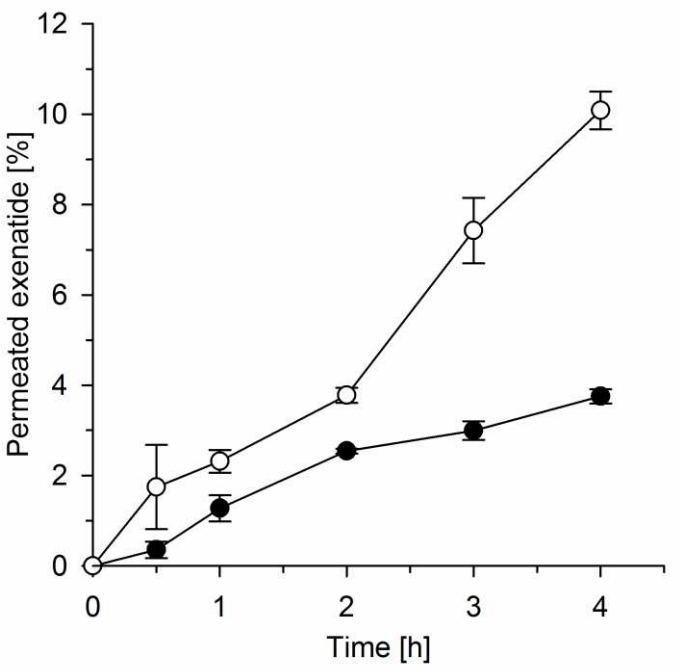

Figure 4: Mucus permeation study using transwell® system over 4 h at 37 °C. Percentage of permeated exenatide as a function of time: exenatide solution (14.3 mg/mL in 50 mM phosphate buffer pH 6.8) (●) and exenatide/DOC SEDDS (○). Values are means of at least three experiments ± SD.

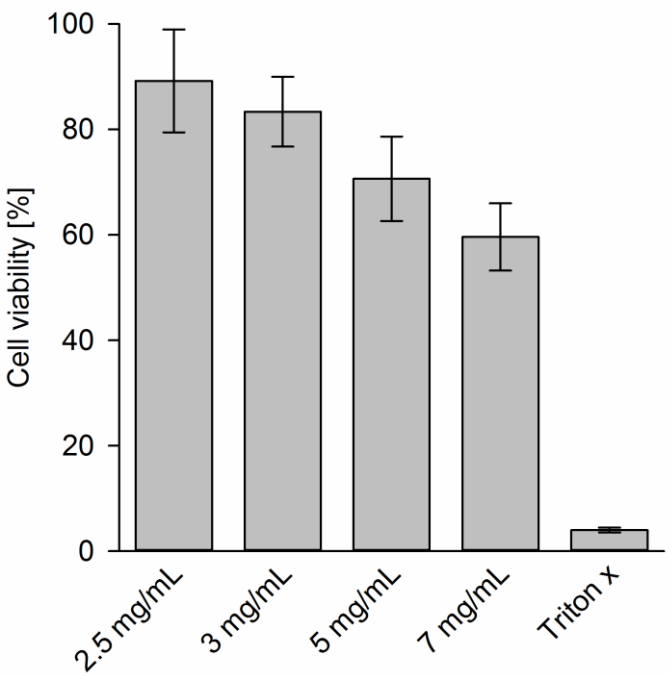

Figure 5: Cytotoxicity of exenatide/DOC SEDDS after 12 h incubation in concentrations of 2.5 mg/mL, 3 mg/mL, 5 mg/mL and **7 mg/mL**~~10 mg/mL~~. Values are means of at least three experiments ± SD.

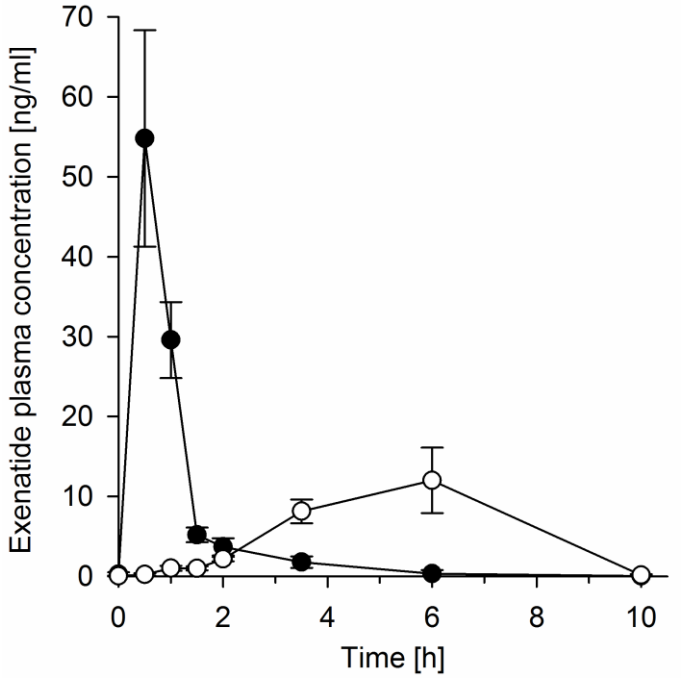

Figure 6: Exenatide plasma concentration-time curve for orally administered exenatide/DOC SEDDS **(dose: 150 µg)** (○) and s.c. exenatide solution **(dose: 20 µg)** (●). Illustrated values are the means of at least three experiments ± standard deviation

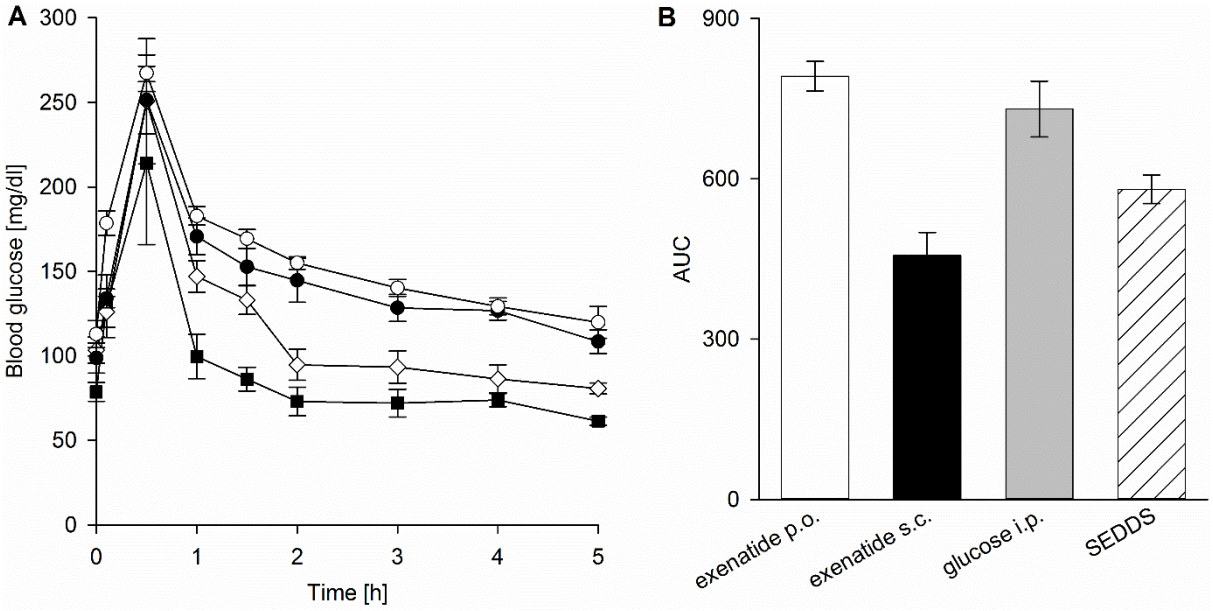

Figure 7 (A+B): In vivo evaluated pharmacodynamics **(A)** Time dependent plasma glucose levels of rats treated with exenatide s.c. + glucose i.p. (■), exenatide/DOC SEDDS p.o. + glucose i.p. (◊), exenatide p.o. + glucose i.p. (○) and glucose i.p. alone (●). **(B)** AUC of plasma glucose levels. Values are means of at least three experiments ± SD.