Oral self-emulsifying delivery systems for systemic administration of therapeutic proteins: Science fiction?

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Thi Nhu Quynh Phan[a,b], Bao Le-Vinha,[c], Nuri Ari Efiana[a,d], Andreas Bernkop-Schnürch[a]*

[a]Department of Pharmaceutical Technology, Institute of Pharmacy, Leopold-Franzens University Innsbruck, Innrain 80/82, A-6020 Innsbruck, Austria

[b] Faculty of Pharmacy, University of Medicine and Pharmacy, Hue University, Vietnam

[c] Department of Industrial Pharmacy, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh city, Vietnam

[d]Department of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Ahmad Dahlan, Jl. Prof. Dr. Soepomo, S.H., Janturan, Warungboto, Umbulharjo, Yogyakarta 55164, Indonesia

Abstract

Objective: The aim of this study was to develop self-emulsifying drug delivery systems (SEDDS) for oral delivery of therapeutic proteins through hydrophobic ion pairing.

Method: Horseradish peroxidase (HRP), a model protein, was ion paired with sodium docusate to increase its hydrophobicity. The formed enzyme – surfactant complex was incorporated into SEDDS, followed by permeation studies across Caco-2 cell monolayer and freshly excised rat intestine.

Results: Hydrophobic ion pairs (HIP) were formed between HRP and sodium docusate with the efficiency of $87.49 \pm 1.35\%$. The formed complex maintained $60.97 \pm 1.48\%$ of the original enzyme activity. The ion pair was subsequently loaded into SEDDS with a payload of 0.1% (mass percent, m/m). The obtained emulsion formed by SEDDS had a droplet size in the range from 20 to 200 nm with negative zeta potential. Permeation mechanism of the enzyme was energy-dependent and the encapsulation of the HIP complex in SEDDS enhanced the permeation of the enzyme through the Caco-2 cell monolayer and freshly excised rat intestine by 4 times and 2.5 times compared to free enzyme, respectively.

Conclusion: According to these findings, hydrophobic ion pairing followed by incorporation to SEDDS might be considered as a potential strategy for oral delivery of therapeutic proteins.

Keywords: Hydrophobic ion pairing, Protein delivery, Self-emulsifying drug delivery system, Horseradish peroxidase

Introduction

Showing great potential as therapeutics, protein drugs have attracted increasing attention as an alternative to conventional small organic drug molecules with hundreds of protein drugs having already reached the market [1]. Although oral administration is the preferred route of drug delivery because of its convenience, low-cost and patient compliance, the commercial success of non-invasive delivery systems for therapeutic proteins remains very limited [2]. High molecular mass, low lipophilicity and charged functional substructures are distinctive properties of protein dugs leading to their poor oral bioavailability. In addition, degradation in the gastro-intestinal (GI) environment and epithelial barriers of the small

*Corresponding author: Andreas Bernkop- Schnürch, Center for Chemistry and Biomedicine, Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innrain 80/82, 6020 Innsbruck, Austria. E-mail address: andreas.bernkop@uibk.ac.at

intestine are also obstacles in oral protein delivery [3]. Strategies to overcome these hurdles focus mainly on the co-administration of permeation enhancers [3-5] and enzyme inhibitors [3, 6].

More recently self-emulsifying drug delivery systems (SEDDS), isotropic mixtures of oils, surfactants, one or more hydrophilic solvents and co-solvents/surfactants which spontaneously emulsify to form fine oil-in-water under mild agitation as in the GI tract, have gained attraction in oral delivery of peptide and protein therapeutics as it turned out that these hydrophilic macromolecular drugs can be incorporated in the oily phase of SEDDS via hydrophobic ion pairing (HIP) [7-13]. During the ion pairing process, charged amino acids are reversibly neutralized by oppositely charged surfactants, increasing the lipophilic character of proteins [14, 15]. HIP may improve the permeation of the protein across GI epithelial membranes as increasing hydrophobicity seems beneficial for transcellular absorption [16]. Due to the significantly increased lipophilic character of these drugs payloads of even 10% in SEDDS were reached [7, 17].

SEDDS per se were shown to have the ability of protecting the incorporated therapeutic molecules towards proteolytic degradation, exhibiting mucus-permeating properties, controlling drug release and consequently improving bioavailability [18]. Formulating SEDDS while maintaining the stability and efficacy of therapeutic proteins, however, remains a challenge [2]. In particular, it is unclear whether protein drugs remain their functions after HIP and incorporation into SEDDS, and whether they can permeate the absorption membrane in sufficient quantities. Complex formation and encapsulation into SEDDS might cause changes in the tertiary structure of the protein, thus its biological activity might be reduced or even lost. It was therefore the aim of this study to investigate whether proteins can maintain their function after HIP and incorporation to SEDDS and whether such systems exhibit sufficiently high membrane permeating properties. As model protein, horseradish peroxidase (HRP) was chosen as this enzyme is very sensitive to conformational changes resulting in a loss of its enzymatic activity [19]. Furthermore, with a size of 44 kDa it is a challenging candidate for intestinal membrane permeation studies.

Materials and Methods

Materials

HRP and sodium docusate were purchased from Alfa Aesar. Capriol 90, Peceol, Labrasol ALF and Labrafil M1944CS were gifts from Gattefossé (France). Capmul MCM EP, Capmul 907P and Capmul PG-8 were kindly donated by Abitec (USA). Sodium deoxycholate, Kolliphor RH40, Kolliphor EL, Tween 80, Tween 40, Tween 20, Brij O10, Transcutol HP and propylene glycol were purchased from Sigma-Aldrich (Austria). Other chemicals were sodium lauryl sulfate and glycerol (Gatt-Koller, Austria), sodium oleate and PEG 300 (TCI Chemicals, Germany), Kolliphor HS15 (BASF, Germany), Miglyol 840 (Sasol, Germany), and Crodamol GTCC (Croda, Germany). All other reagents were of analytical grade and purchased from SigmaAldrich, Austria.

Enzymatic activity assay

Peroxidase activity was determined according to a previously reported assay in phosphate buffer (200 mM, pH 6.5) [20] with some modifications. Briefly 50 µl of HRP dissolved in demineralized water or withdrawn aliquots from the studies described below was transferred to a 96-well microtitration plate and diluted in a ratio of 1:3 by the addition of 100 µl of 200 mM phosphate buffer pH 6.5. Then 50 µl of substrate solution consisting of 24 mg of o-phenylendiamine dihydrochloride, 2.4 ml of 1 M phosphate buffer pH 6.5, 9.6 ml of demineralized water and 24 µl of 30% H_2O_2 was added. The enzymatic reaction was allowed to proceed at room temperature for 5 min and was stopped by the addition of 50 µl of 2 M HCl. Absorbance of the reaction mixtures was measured at 492 nm with Tecan infinite® M200 spectrophotometer, Austria.

Effect of surfactants on ion pairing

Four anionic surfactants namely sodium lauryl sulfate, sodium oleate, sodium deoxycholate and sodium docusate were utilized to precipitate HRP. Surfactant solutions (500 µl, 1.5 mM) were dropwise added to HRP solution (500 µl, 1.5 mg/ml) at room temperature. The resulting precipitate was isolated by centrifugation at 12,100 g for 5 min (MiniSpin®, Eppendorf, Austria). The degree of HRP-surfactant ion pairing was determined by quantifying the enzyme

remaining in the supernatant utilizing the assay as described above. Enzyme solution without surfactant and demineralized water served as control and blank, respectively, considering the specificity of the assay to HRP, the low concentration of the unreacted surfactant and their negligible absorbance at 492 nm. The precipitation efficiency was calculated utilizing the following equation:

Ion pairing efficiency (%)=100-(Enzyme concentration after HIP)/(Enzyme concentration before HIP) x 100

The obtained precipitate was washed twice with demineralized water, lyophilized (Christ Gamma 1-16 LSC Freeze dryer) and stored at -20oC until further use.

Effect of surfactant concentration and pH on ion pairing

The effect of different HRP-surfactant ratios on the ion pairing efficiency was examined to identify the most appropriate ratio. HRP solution (1.5 mg/ml) was adjusted to pH 2.5 with 2 M hydrochloric acid solution. Sodium docusate water solution (500 μL) was added dropwise to HRP solution (500 μL). Different molar ratios of HRP to sodium docusate were tested (1:15, 1:30, 1:45, 1:60, 1:75, 1:90 and 1:105). The resulting precipitate was isolated by centrifugation at 12,100 g for 5 min (MiniSpin®, Eppendorf, Austria) and ion pairing efficiency was determined by quantifying the enzyme remaining in the supernatant utilizing the assay as described above. Zeta potential of the resulting precipitates was measured right after they were formed and homogenously dispersed in the resulting solutions utilizing Zetasizer Nano ZSP (Malvern Instruments Ltd, Worcestershire, UK). To investigate the effect of pH on the ion pairing process, sodium docusate (1.5 mM) was paired with HRP (1.5 mg/ml) at pH of 2.5, 3.0, 3.5 and 4.0. Ion pairing efficiency was determined by quantifying the formation of HRP-surfactant complex.

SEDDS development and characterization

Based on preliminary studies on solubility and stability of HIP in different solvents and surfactants, SEDDS formulations were prepared with Capmul MCM EP as solvent, propylene glycol as co solvent and Tween 40, Kolliphor RH40 or Kolliphor HS15 as surfactants. The components were mixed in ratios listed in Table 1 and homogenized at 25 oC and 1200 rpm using a thermomixer (Thermomixer®,Eppendorf, Austria). For each experiment, SEDDS formulations of 1 g were prepared. In case of HIP loaded formulations, the complex was first dissolved in propylene glycol and subsequently added to the premixed solvent and surfactant mixtures. SEDDS preconcentrates were diluted 1:100 in 50 mM phosphate buffer pH 6.8 and the resulting emulsion was characterized in terms of mean droplet size, polydispersity index and zetapotential using Zetasizer Nano ZSP (Malvern Instruments Ltd, Worcestershire, UK). Additionally, stability of SEDDS formulations and dispersion was visually evaluated in terms of incorporated complex precipitation and phase separation after storage time of 24 h and 48 h at room temperature.

Enzymatic activity of HRP after complexation and loading to SEDDS

Enzymatic activity of HRP after complexation and loading to SEDDS was determined by the above described assay. Specifically, HRP-docusate ion pair was dissolved in 10 mM sodium dihydrogen phosphate and mixed vigorously to dissociate. Similarly, SEDDS formulations were dispersed in 10 mM sodium dihydrogen phosphate at a concentration of 0.01% (m/v), followed by centrifugation at 12,100 g for 15 min (MiniSpin®, Eppendorf, Austria). Fifty microliters of the resulting solutions was transferred to a 96-well microtitration plate and proceeded to the enzymatic activity assay described above. Absorbance of the reaction mixtures was measured at 492 nm with Tecan infinite® M200 spectrophotometer, Austria.Enzymatic activity of the resulting solutions was determined and compared with HRP solutions of equal concentration.

Cell viability assessment

To examine the cytotoxic potential of HIP loaded SEDDS, resazurin assay was performed on Caco 2 cells. Caco-2 cells were cultured on a 24 well plate (d = 2.5 ⬜ 104 cells/well; 500 μl per well) in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (100 units/0.1 mg/L) for 14 days at 37 oC in an

atmosphere of 5% CO2 and 95% relative humidity. The medium was changed every other day during the culture period. On the day of experiment, cells were washed with pre-warmed Hank's balanced salt solution (HBSS, pH 7.4). SEDDS dispersions at concentrations of 0.3% and 0.5% (m/v) in HBSS were added (500 µl/well) and the cells were incubated for 4 h at 37oC. MEM without phenol red and 0.1% (m/v) Triton X-100 in HBSS served as positive and negative control, respectively. After the incubation, SEDDS dispersions were removed. Cells were washed twice with HBSS, followed by the addition of 250 µl of 44 µM resazurin in phosphate buffer saline. Cells were further incubated for 3 h. The supernatants were transferred to a 96-well plate and their fluorescence intensity were measured at the excitation wavelength of 540 nm and the emission wavelength of 590 nm (Tecan infinite® M200 spectrophotometer, Austria). Cell viability was calculated as follow:

Cell viability (%)=Fs/Fc x 100

where Fs = fluorescent intensity of SEDDS dispersion, Fc = fluorescent intensity of negative control.

Permeation study across Caco-2 cell monolayer

Permeation studies on Caco-2 cells were performed as previously described with some modifications [21, 22]. Caco-2 cells were seeded onto 24-well Cellstar cell culture plates with ThinCertTM inserts (Greiner bio-one, 0.4 mm pore size) at the density of 0.6×10^5 cells/cm2. Cells were cultured in supplemented MEM at 37 °C in an atmosphere of 5 % CO2 and 95 % humidity. The culture medium was changed every other day and cells were allowed to grow and differentiate for 21 days. On the day of experiment transepithelial electrical resistance (TEER) of the cell monolayer was measured with the EVOM instrument (World Precision Instruments, Sarasota, FL, United States). Only cell monolayers with TEER values in the range of 300-500 Ω.cm2 were proceeded to the permeation study. In order to evaluate the integrity of the cell monolayer after the study TEER was measured subsequently. HBSS was used as permeation medium. Prior to all experiments, cells were washed and equilibrated with HBSS for 30 min, followed by the addition of 500 µl of HBSS to the

acceptor chamber and 100 µl of SEDDS dispersed in HBSS at the concentration of 0.3% (m/v) to the donor chamber. HRP in HBSS at the same concentration as that of SEDDS served as control, whereas HBSS alone served as blank. The study was performed at 4 °C and 37 °C to evaluate the effect of temperature on the permeation. After 30, 60, 90, 120 and 180 min, 50 µl of aliquots were withdrawn from the acceptor chamber and replaced by an equal volume of pre-warmed HBSS. The amount of permeated HIP was analyzed by the enzymatic assay described above and cumulative corrections were made for the previously removed aliquots. The apparent permeability coefficients (Papp) for SEDDS were calculated according to the following equation:

$$Papp = Q/(A*C_0*t)$$

where Papp is the apparent permeability coefficient (cm/s), Q is the total amount permeated throughout the incubation time (nmol), A is the area of the insert (cm^2), C_0 is the initial concentration of HIP in the donor compartment (nmol/cm^3) and t is the time of permeation study (s). The experiment was performed in triplicate.

Permeation across freshly excised rat intestine

For the ex-vivo permeation studies, small intestine of male Sprague–Dawley rats (weighing between 200 and 300 g) was immediately removed after sacrificed and placed in normal saline. Distal jejunum and ileum segments from excised intestine was cut into strips of 1.5 cm, washed to get rid of luminal contents and mounted in the Ussing-type chambers (0.64 cm2 surface area). Apical and basolateral chamber was filled with 1 ml of HBSS. Ussing chambers were placed in a water bath maintained at a temperature of 37 oC. After pre-incubation for 30 min, medium of the luminal side of chamber was replaced with SEDDS dispersed in HBSS at the concentration of 0.3% (m/v) or HRP only in HBSS at the same concentration. As a control for the presence of endogenous peroxidase activity, Ussing chambers with HBSS alone were also incubated and served as blank. Over a period of 3 h, 50 µl of aliquots were withdrawn from basolateral compartment after every 30 min and were replaced by the same amount of fresh HBSS maintained at 37 oC. The amount of samples

permeated through the tissue was determined and calculated as described above.

Statistical analysis

Statistical data analysis was performed using two-tail student t-test with $p < 0.05$ as the minimal level of significance. All values were expressed as the means ± SD of at least three replications

Results and Discussion

Hydrophobic ion-pairing

Among the tested surfactants as illustrated in Figure 1, sodium oleate and sodium deoxycholate failed to precipitate the enzyme from solution. In contrast, sodium docusate and sodium lauryl sulfate showed high precipitation efficiency of 87.49 ± 1.35% and 60.71 ± 4.40%, respectively, when they were applied in a concentration of 1.5 mM corresponding to the ratio of 1:45. The fact that sodium docusate provided the most efficient ion pairing with HRP confirmed its role as a universal counter ion [7]. HRP - docusate complex was therefore chosen for further studies.

Different molar ratios of enzyme to sodium docusate were tested revealing the ratio of 1:45 as the most efficient (Figure 2A). When more surfactant was added, the amount of HIP precipitate steadily decreased as more HRP was detected in the supernatant. The result indicated that the surfactant was unlikely to be associated with a deteriorating effect on HRP activity. The observation was also in good agreement with previous studies showing lower precipitation efficacy beyond the stoichiometric ratio [17, 23]. The decrease in precipitation can be explained by the formation of micelles and the solubilizing properties of excess surfactant. In addition to the effect of HRP - surfactant ratio on the complex formation efficiency, a shift in zeta potential during HIP was observed. This effect is illustrated in Figure 2B. In general the more docusate was added to the enzyme solution, the more negative was the zeta potential of the resulting complex. Below the molar ratio of 1:45, zeta potential values were around zero ranging from -10 to +10 mV. Addition of surfactant at higher molar ratios resulted in a decrease in zeta potential values, for instance, to about -20 mV at HRP-docusate ratio of 1:60. Further increased concentrations of

sodium docusate led to minor changes in zeta potential indicating a likely constant amount of surfactant molecules in the enzyme-surfactant complex. This observation was in agreement with previous studies performed by our research group [7,17].

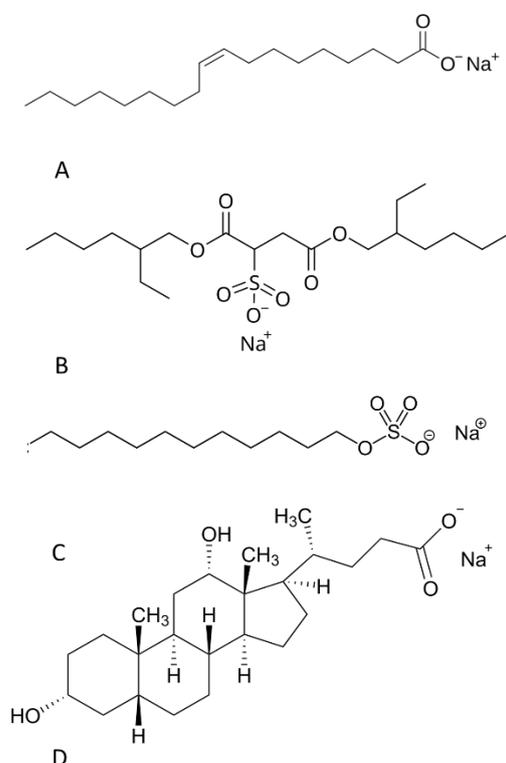Amino acid sequence of horseradish peroxidase with positively charged subdomains

Figure 1. Amino Acid sequence of HRP and molecular structure of tested surfactants for HIP formation of HRP, A: sodium oleate, B: Sodium lauryl sulphate, C: sodium docusate, D: sodium deoxycholate

HRP consists of a number of distinctive peroxidase isoenzymes [24, 25]. Therefore, the enzyme product has a broad range of isoelectric point values ranging from 3 to 9 depending on the composition of the isoenzymes. Among the various isoenzymes, isoenzyme C (HRP C) is the most abundant one with an isoelectric point close to neutral [25, 26]. Therefore, HRP solutions of

different pH values ranging from 2.5 to 4.0 were tested regarding ion pairing efficiency. The results were illustrated in Figure 3. The highest precipitation efficacy was obtained at pH 2.5 with 87.49 ± 1.35%. Increasing the pH from 3.0 to 4.0 led to a decrease in the ion pairing efficiency from 48.7 ± 5.3% to 10.8 ± 5.4%. Consequently, pH 2.5 was chosen as the most appropriate condition for ion pair formation.
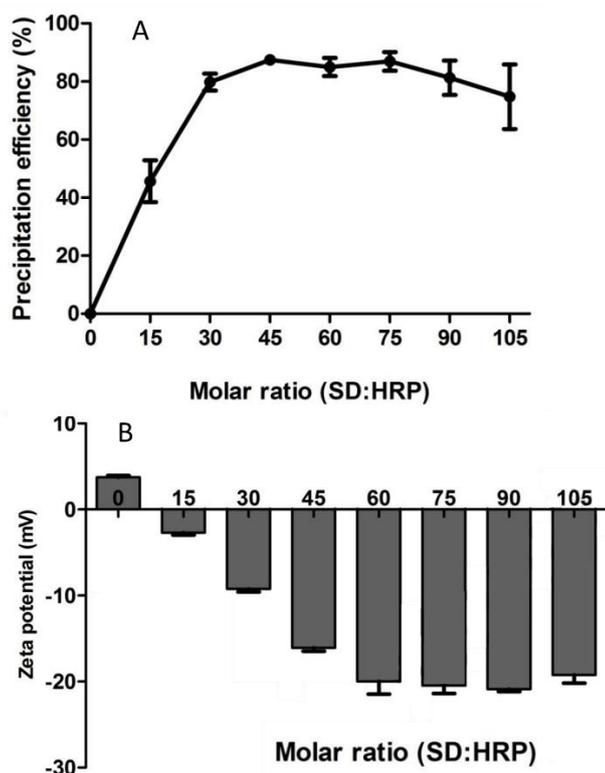

Figure 2. The effect of SD to HRP molar ratio on precipitation efficiency (A) and Zeta potential of precipitate resulting from HIP formation of HRP-Docusate complex. Data are shown as mean ± SD (n= 3).
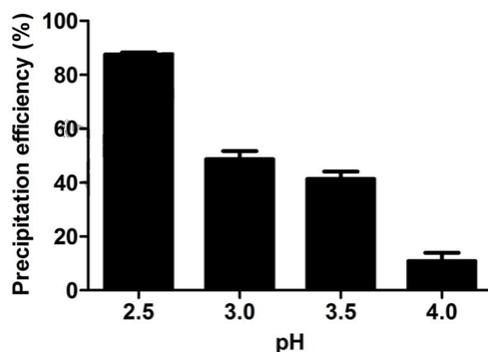

Figure 3. Effect of pH on precipitation efficiency in the formation of HRP-Docusate complex at a molar ratio of 1:45. Data are shown as mean ± SD (n= 3).

SEDDS development and characterization

The formation of HIP significantly enhanced hydrophobicity of HRP reflected by the very poor water solubility of 0.004 % (mass percent, m/m) compared to the original enzyme. Based on preliminary studies on the solubility and stability of HIP in different solvents, co-solvents and surfactants (data not shown), Three SEDDS formulations as listed in Table 1 were prepared and the HRP-docusate complex was incorporated in a concentration of 0.1% (m/m). The resulting pre-concentrates spontaneously formed emulsions upon dilution with 50 mM phosphate buffer pH 6.8 in a ratio of 1:100 (m/v). Size distribution and zeta potential were recorded for blank SEDDS and for HRP-docusate loaded SEDDS (Table 1). Both loaded and blank SEDDS exhibited droplet size distribution in the range between 20 and 200 nm and the loading of HIP had only a minor impact on droplet size. HRP complex loaded SEDDS exhibited a more negative zeta potential compared with blank SEDDS, which might be due to the anionic amino acids of HRP exposed on the surface of the droplets. A slightly negative charge might be advantageous as the oily droplets on the one hand can permeate more easily the negatively charged mucus layer and provoke on the other hand not too high electrostatic repulsion forces with the negatively charged cell membrane [17, 27]. The newly developed SEDDS also showed sufficient stability as neither precipitation of the HRP complex from SEDDS pre-concentrates and dispersions nor phase separation of SEDDS was observed after 24 h and 48 h at room temperature.

Enzymatic activity of HRP after complexation and loading to SEDDS

As the complex has low solubility in water, a basic salt solution was used to foster the enzyme release from the complex. SEDDS formulations were dispersed at a high dilution ratio of 1:10,000, followed by centrifugation in order to release loaded HIP from SEDDS for measuring enzymatic activity. After liberation from HIP, a remaining activity of 60.97% ± 1.48 was determined suggesting that HRP is still available in its native structure (Figure 4). Nevertheless, there was a further decrease of activity after liberated from SEDDS in comparison to a control of native

Table 1. Composition of SEDDS pre-concentrates (%, m/m) and characterization of blank SEDDS and SEDDS loaded with horseradish peroxidase (HRP)–docusate complex. Data are shown as mean ± SD (n= 3).

Formu lation	HRP-docusate complex	Propy lene glycol	Capmul MCM	Tween 40	Kolli phor RH40	Kolli phor HS15	Particle size (nm)		Zeta potential (mV)	
							Blank SEDDS	Loaded SEDDS	Blank SEDDS	Loaded SEDDS
FI	0.1	30	25	45	-	-	180.73 + 1.74	185.15 + 5.30	-10.52 + 0.77	-12.42 + 0.25
FII	0.1	30	25	-	45	-	20.74 + 1.13	21.34 + 0.50	-3.22 + 0.57	-4.75 + 0.25
FIII	0.2	30	25	-	-	45	172.60 + 2.38	177.57 + 2.58	-6.47 + 0.22	-10.23 + 0.30

enzyme. The remaining activities were around 30% for FII and under 5% for FI and FIII. One possible explanation for the decrease in activity of the enzyme is the successive change of the pH during ion pairing and complex dissociation. Besides, interactions with docusate may destabilize the enzyme by triggering conformational change or partial unfolding, resulting in a decrease in enzymatic activity compared to native HRP [15]. Moreover, enzymes need a hydrophilic environment to express their activities, thus an increase in hydrophobicity of the surrounding media could result in lower activity as observed after loading HIP to SEDDS [9]. The observation was also in agreement with the preliminary study in which the stability profile of the complex varied according to surfactants and surfactant concentrations. However, as HRP is a comparatively labile coenzyme and prone to rapid conformational changes the determined enzymatic activity of the HRP-surfactant complex and SEDDS formulation FII provides evidence for the practical relevance of this technique.
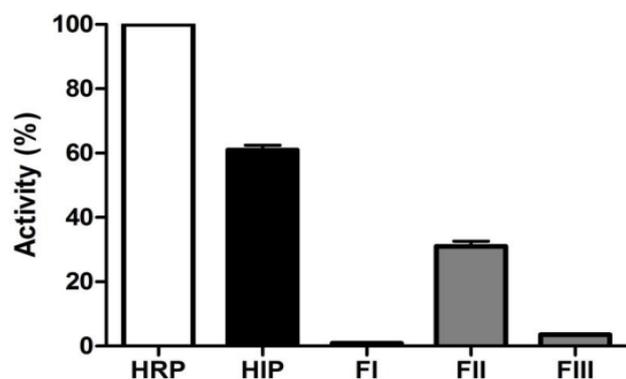

Figure 4. Peroxidase activity of native enzyme (HRP), HRP-docusate complex (HIP), HRP-docusate complex released from SEDDS formulation. Data are shown as mean ± SD (n= 3).

Cell viability assessment

SEDDS is a mixture of lipids, surfactants and/or co-solvents whose potential toxic effect on enterocytes was reported in previous studies. A dose-dependent cytotoxic effect of commonly used lipids (medium-chain mono- and tri-glycerides) and non-ionic surfactants (Tween 80 and Cremophor EL) on Caco-2 cells was, for instance, described by Bu et al. [28, 29]. A dose-dependent cytotoxic effect of polyethylene glycol ester and propylene glycol ester surfactants on Caco-2 cells was also shown in a concentrationdependence as well [30]. In this study, cell viability was evaluated at two concentrations of SEDDS, i.e. 0.3% and 0.5% (m/v). Formulations FI and FII appeared to be non-toxic at 0.5% (m/v), whereas FIII was more toxic at this concentration in which cell viability was even halved. At lower concentration of 0.3% (m/v), all formulations were showed to be safe to the cell monolayer as almost no cytotoxic effect was observed (Figure 5). Based on enzyme activity study and cytotoxicity evaluation, FII was selected for further studies.

Permeation study across Caco-2 cell monolayer

Caco-2 cell model is the most extensively characterized and used test system in the field of drug permeability studies [31]. When grown to confluence, cell polarity and tight junctions are established and several active transport systems are expressed mimicking human intestinal epithelium. Papp measured for certain compounds across Caco-2 cell monolayer have shown good correlation with in vivo absorption [32]. As a result, Caco-2 cell monolayer has been considered as a standard in vitro model for

assessing the intestinal permeability and transport of drug candidates and lead compounds [33].
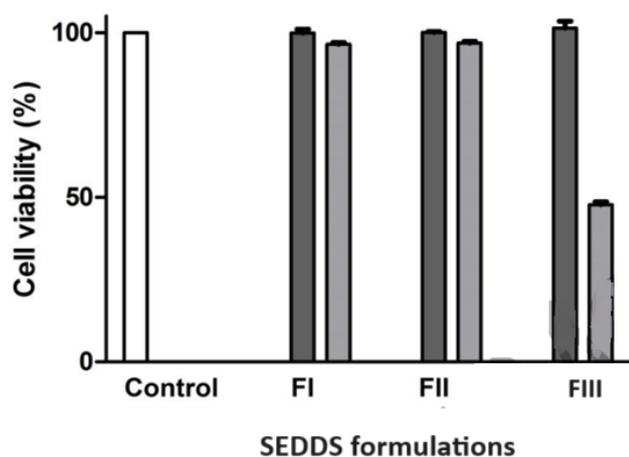

Figure 5. Cytotoxicity assay of formulation against Caco2 cell for 4 h of incubation. The concentrations of SEDDS were 0.3% (black bar) and 0.5% (grey bar). Data are shown as mean ± SD (n= 3).

It has been accepted that highly absorbed drugs have Papp of higher than 1×10^{-6} cm/s whereas incompletely absorbed drugs have Papp of lower than 1×10^{-7} cm/s on Caco-2 model [32]. According to this, FII showed an intermediate permeability with Papp of 0.54×10^{-6} cm/s while HRP had rather low permeability with Papp of only 0.13×10^{-6} cm/s at 37 °C (Figure 6). The permeation of FII was 4-fold higher than that of HRP solution, which demonstrated the improvement in permeation of the enzyme after incorporation into SEDDS. The enhanced permeation might be due to the increase in hydrophobicity of the enzyme through complexation with the surfactant facilitating the absorption across the lipid membrane when the complex is released at the epithelium.

In previous study on a plasmid DNA - cationic lipid complex, the presence of the plasmid DNA was found in 100% of the cells indicating the uptake enhancing effect due to the increase in hydrophobicity of the complex [34]. Lipidization was also shown to significantly enhance cellular uptake of leu-enkephalin in Caco-2 cells in a study of Wang et al. [35]. The permeation enhancing effect might also result from SEDDS with the presence of surfactants in the formulation and the increased surface area by the nano-sized SEDDS droplets. Two surfactants Kolliphor RH40 and docusate were present in the formulation. As Kolliphor RH40 is not known as a permeation

enhancer and the concentration of docusate in permeation studies was as low as 0.00009% (m/v), however, a permeation enhancing effect of these surfactants was can be excluded. The comparatively low concentration of docusate used in HIP illustrated also the great potential of this technology in comparison to the co-administration of permeation enhancers that were in most cases also surfactants but showing only in at least 10,000-fold higher concentration an improved drug uptake. It was also observed that at lower temperature of 4 °C, Papp of FII significantly decreased to 0.01×10^{-6} cm/s whereas free enzyme showed no permeation at all. The low permeation observed at 4 °C suggested that the permeation process of the enzyme through Caco-2 cell monolayer was energy-dependent [36]. The existence of an energy-dependent transcellular pathway in the total HRP fluxes across Caco-2 monolayer was also reported by Heyman et al. [37]. In this study, as the permeation was quantified by utilizing enzymatic assay, the enzyme appeared to maintain activity after crossing the cell monolayer.
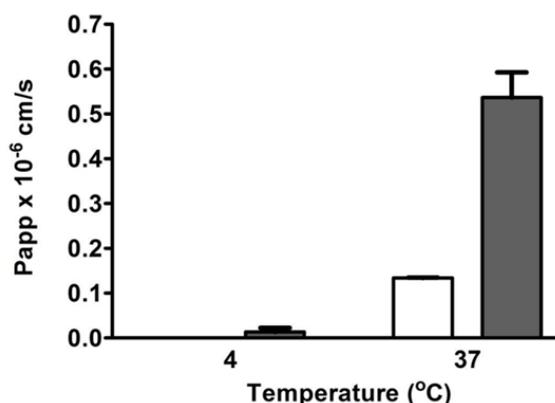

Figure 6. Caco2 cell permeability of nanodroplets containing HRP-docusate complex FII (black bar) in comparison to HRP solution (white bar). Data are shown as mean ± SD (n= 3).

Permeation study across freshly excised rat intestine

Although developed over 70 years ago, the Ussing chamber is still a valuable, time-proven method for the measurement of electrolyte, nutrient, and drug transport across epithelial tissues [38]. There has been good correlation between rat jejunum permeability and human jejunum absorption of several compounds after oral drug administration [39, 40]. Therefore, permeation study on freshly excised rat small

intestine by the Ussing chamber method can be used with decision to predict in vivo oral absorption in human [39]. In our study, FII and HRP showed high permeation through freshly excised rat intestine with Papp of 1.48×10^{-6} cm/s and 0.60×10^{-6} cm/s, respectively (Figure 7). Compared to Caco-2 cell monolayer, FII displayed a 3-fold increase in permeation whereas the free enzyme experienced a 4.5-fold enhancement in permeation across freshly excised rat intestine.
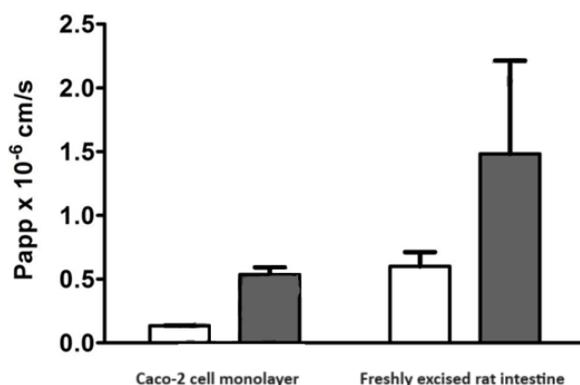

Figure 7. Comparison of the permeability of nanodroplet containing HRP-Docusate complex of FII (black bar) and HRP solution (white bar) across Caco2 cell monolayer and excised rat intestine. Data are shown as mean ± SD (n= 3).

The results were consistent with the findings of previous studies in which Caco-2 model displayed low permeability to the non-specific transepithelial passage of macromolecules, specifically filter-grown Caco-2 cells were relatively impermeable to the transport of HRP [41]. In contrast, the absorption of HRP was observed in the adult jejunum ileum of rats [42, 43] and the enzyme could be detected within lumina of blood and lymphatic capillaries, which strongly suggested the possibility of transportation of HRP from the intestine to the circulation [44]. It was also noticeable that the permeation of FII was 2.5 times higher than that of free enzyme across rat intestine. As reversible lipidization was reported to enhance the absorption of salmon calcitonin and leu-enkephalin across intestinal mucosa reflected by higher plasma concentration after oral administration to experimental animals [35, 45], the increased permeation of the enzyme may be explained by the hydrophobization through hydrophobic ion pairing. This result together with the obtained data from Caco-2 cells model confirmed the ability of HIP to enhance absorption across epithelial membrane

Conclusion

In this study, a potential SEDDS for oral protein therapeutics delivery was developed. Utilizing hydrophobic ion pairing HRP was successfully incorporated into the delivery system. The process of complexation and encapsulation did not cause a deleterious effect on the enzymatic activity of HRP. Furthermore, permeation studies on Caco-2 cell monolayer and rat intestine showed that the HIP loaded SEDDS was able to deliver the enzyme across the intestinal membrane while remaining its activity. According to these results, SEDDS in combination with HIP technique can be regarded as promising tools for oral protein drug delivery giving reason for hope that the oral administration of therapeutic proteins is not anymore science fiction.

Acknowledgments

Conflict of interest

The authors report no conflicts of interest

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