

## Regular Article

# Trypsin decorated self-emulsifying drug delivery systems (SEDDS): Key to enhanced mucus permeation



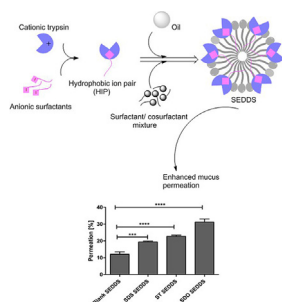
Iram Shahzadi<sup>a</sup>, Aida Dizdarević<sup>a</sup>, Nuri Ari Efiana<sup>a,b</sup>, Barbara Matuszczak<sup>c</sup>,  
Andreas Bernkop-Schnürch<sup>a,\*</sup>

<sup>a</sup> Center for Chemistry and Biomedicine, Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innrain 80/82, 6020 Innsbruck, Austria

<sup>b</sup> Department of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Ahmad Dahlan, Jl Prof Dr Soepomo, S.H., Janturan, Warungboto, Umbulharjo, Yogyakarta 55164, Indonesia

<sup>c</sup> Center for Chemistry and Biomedicine, Department of Pharmaceutical Chemistry, Institute of Pharmacy, University of Innsbruck, Innrain 80/82, 6020 Innsbruck, Austria

## GRAPHICAL ABSTRACT



## ARTICLE INFO

## Article history:

Received 4 June 2018

Revised 12 July 2018

Accepted 14 July 2018

Available online 17 July 2018

## Keywords:

Hydrophobic ion pairing

Lipophilicity

Mucus permeation

Trypsin

Self-emulsifying drug delivery system

(SEDDS)

## ABSTRACT

It was the aim of this study to prepare trypsin decorated mucus permeating self-emulsifying drug delivery systems (SEDDS). Lipophilicity of enzyme was increased by hydrophobic ion pairing (HIP) with the anionic surfactants sodium dodecyl sulfate (SDS), sodium taurocholate (ST) and sodium deoxycholate (SDO) to facilitate its incorporation in SEDDS. Blank SEDDS and trypsin decorated SEDDS were characterized regarding droplet size, polydispersity index (PI), zeta potential and proteolytic activity using  $N\alpha$ -benzoyl-L-arginine ethyl ester (BAEE) assay.  $\log D_{\text{SEDDS}/\text{release medium}}$  of each complex was determined to assess its affinity towards SEDDS oily droplet upon emulsification. Ability of trypsin decorated SEDDS to enhance mucus permeation was studied on mucus gel from porcine small intestine for the period of 4 h at 37 °C. Degree of enzyme precipitation via HIP was 94.5%, 85.7% and 48.2% for SDS, ST and SDO complex, respectively. SEDDS composed of 50% (w/w) cremophor EL, 20% (w/w) captex 300, and 30% (w/w) propylene glycol with a complex payload of 1% (w/w) exhibited a droplet size in the range of  $29.92 \pm 0.09$  nm to  $39.15 \pm 0.37$  nm, a polydispersity index of 0.116–0.265 and zeta potential in the range of  $-2.36$  mv to  $-4.25$  mv. The enzymatic activity of trypsin complexed with SDO, SDS and ST in SEDDS was 51.9%, 44.8%, and 40.7% respectively, of the corresponding activity of free trypsin.  $\log D$  values of trypsin, SDS, ST and SDO complex were  $-2.73$ , 1.97, 1.89 and 1.68, respectively, suggesting higher lipophilicity of trypsin complexes as compare to free trypsin and ability to reside on SEDDS droplets. Enzyme decorated SEDDS improved mucus permeation 1.6- to 2.6-fold in comparison to blank SEDDS. Results demonstrated that decorating SEDDS with trypsin can be a promising technique to improve their mucus permeating properties.

© 2018 Elsevier Inc. All rights reserved.

\* Corresponding author.

E-mail address: [andreas.bernkop@uibk.ac.at](mailto:andreas.bernkop@uibk.ac.at) (A. Bernkop-Schnürch).

## 1. Introduction

Mucus is a viscoelastic hydrophilic gel layer covering the mucosal surfaces of the body where it lubricates and protects the underlying epithelium from toxic substances and pathogens, while enabling the exchange of nutrients [1]. It consists of mainly the water (about 90%) and a small amount of salts, lipids, carbohydrates and mucin (negatively charged glycoproteins) [2]. Most foreign molecules are efficiently trapped within the mucus gel layer by steric obstruction and adhesion and are cleared from the mucosal surface within seconds to a few hours depending on their location sites [3]. Mucus has also been recognized as a strong barrier to drug diffusion such as polypeptides with a molecular mass higher than 12.4 kDa [4,5]. Accordingly innovative drug carriers are needed that can overcome this barrier to improve drug absorption. Among several strategies like the use of PEGylated nanoparticles [6], highly densely charged nanoparticles [7] and proteolytic enzyme decorated nanoparticles [8], one promising strategy seems to be the decoration of self-emulsifying drug delivery systems (SEDDS) with mucolytic enzymes to enhance mucus permeation. SEDDS are isotropic mixtures of oil, surfactant and co-surfactant spontaneously forming an O/W nano-emulsion upon mixing with water. These are one of the most promising strategies to resolve solubility and bioavailability issues in drug delivery [9]. Potential advantages of SEDDS are lower interactions with mucus likely due to favorable surface properties and simple production and scale up process [10]. In a previous research study, papain loaded mucolytic SEDDS provided promising results to enhance mucus permeation [11]. As papain is an exogenous enzyme obtained from the juice of the papaya fruit [12], it might stimulate the immune system provoking allergic reactions. However, endogenous proteolytic enzymes such as trypsin are therefore a more promising option. Its mucolytic action on mucus, sputum and other secretions has already been reported [13,14].

It was, therefore, the aim of this study to decorate SEDDS with trypsin and to evaluate mucus permeation properties of these systems through porcine intestinal mucus. In order to attach trypsin on the surface of SEDDS, hydrophobic ion pairs (HIP) of the enzyme with likely suitable anionic surfactants as illustrated in Table 1, were generated to introduce a lipophilic substructure for anchoring on the oily droplets. Trypsin decorated SEDDS were characterized regarding droplet size, polydispersity index, enzymatic activity and mucus permeation.

## 2. Materials and methods

### 2.1. Materials

Trypsin from porcine pancreas (1459 BAEE units/mg), sodium dodecyl sulfate (SDS,  $\geq 99\%$ ), sodium deoxycholate (SDO,  $\geq 97\%$ ), propylene glycol (PG,  $\geq 99.5\%$ ), sodium phosphate monobasic  $\geq 99\%$ , potassium dihydrogen phosphate  $\geq 99.5\%$ , sodium chloride  $\geq 99\%$ , N $\alpha$ -benzoyl-L-arginine ethyl ester hydrochloride (BAEE)  $\geq 99.5\%$ , sodium hydroxide  $\geq 97\%$ , Fluorescein 5(6) - isothiocyanate mixed isomer (FITC, 90%), fluorescein diacetate (FDA, 98%) and Bowman-Birk inhibitor (BBI 82% protein biuret) were obtained from Sigma-Aldrich Chemie GmbH, Vienna, Austria. Sodium taurocholate (ST,  $\geq 95\%$ ) was purchased from Tokyo Chemical Industry Co., Ltd. Captex<sup>®</sup> 300 (glyceryl tricaprilate/tricaprate,  $\approx 70\%$ ) was obtained from ABITEC Corporation Columbus, USA. Kolliphor EL (Cremophor EL, purity meets Ph. Eur.) was from BASF SE Ludwigshafen am Rhein, Germany. To collect fresh mucus, porcine intestine was obtained from slaughter house, Innsbruck, Austria.

### 2.2. Methods

#### 2.2.1. Hydrophobic ion pairing (HIP)

Three different anionic surfactants, sodium dodecyl sulfate (SDS), sodium deoxycholate (SDO) and sodium taurocholate (ST) were used for hydrophobic ion pairing of trypsin adapting the method described by Lechner et al. [11]. Surfactant solutions were added dropwise to trypsin solution in 0.1 M HCl at room temperature under gentle stirring at trypsin/surfactant molar ratios of 1:2, 1:3, 1:5, 1:6, 1:7, 1:8, 1:9 and 1:10 covering the range around the theoretical molar ratio of 1:7 required for lipidization of all available cationic substructures on trypsin. Solutions were continuously stirred for 2 h. The precipitated complexes were separated by centrifugation at 13,400 rpm for 20 min and washed three times using purified water. Remaining water was removed by freeze drying (Christ Gamma 1–16 LSC Freeze dryer) and complexes were stored at  $-20\text{ }^{\circ}\text{C}$  until use.

#### 2.2.2. Precipitation efficiency

Precipitation efficiency at various trypsin to surfactant ratios was determined by quantifying the remaining enzyme in supernatant after centrifugation with a UV spectrophotometer (UVmini-1240, SHIMADZU). The values were calculated from a calibration curve which was constructed using concentration range 15–750  $\mu\text{g}/\text{ml}$  of trypsin at the  $\lambda_{\text{max}}$  of 210 nm. Percentage precipitated enzyme at each ratio was determined using the following equation as described by Griesser et al. [15]:

$$\text{Precipitated enzyme}(\%) = 100 - \left[ \frac{\text{Trypsin conc. after HIP}}{\text{Trypsin conc. before HIP}} \times 100 \right]$$

#### 2.2.3. Fourier transform-infrared spectrometry

Trypsin and trypsin complexes were characterized by Fourier transform infrared (FTIR) spectroscopy. IR spectra were recorded on a Bruker ALPHA FT-IR apparatus equipped with a Platinum ATR (attenuated total reflection) module.

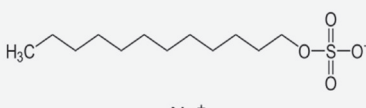
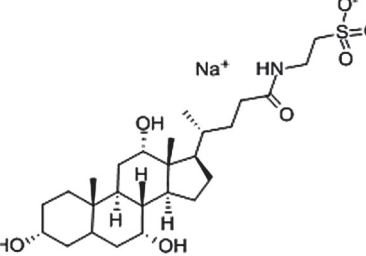
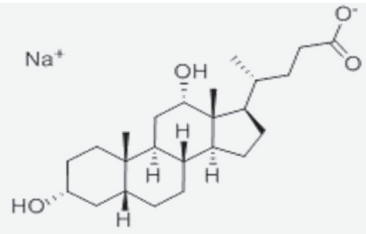
#### 2.2.4. Determination of partition coefficient/ log P values

For determination of log P, a previously described method with some minor changes was used [15]. For this, trypsin and each complex was dissolved in 200  $\mu\text{l}$  of 1:1 octanol and water mixture at  $25\text{ }^{\circ}\text{C}$  for 24 h. After dissolution phases were separated by centrifugation at 13,400 rpm for 10 min using high speed mini centrifuge (Fisher Scientific, Illinois USA) and 50  $\mu\text{l}$  aliquots were taken from each phase. Octanol samples were diluted with methanol and water samples were diluted with water up to total volume of 500  $\mu\text{l}$ . Absorbance of each sample was measured at 210 nm for trypsin, 280 nm for SDS complex, 275 nm for SDO complex and 279 nm for ST complex. Thereafter, log P values were calculated as logarithm of the ratio of concentration in oil phase and that in aqueous phase.

#### 2.2.5. Development and characterization of SEDDS

For the preparation of SEDDS, Captex<sup>®</sup> 300 as oil, Kolliphor EL as surfactant, propylene glycol (PG) as co-surfactants were homogenized in concentrations as listed in Table 2 using vortex mixer (neoLab<sup>®</sup> D-6012). Each complex was incorporated up to 1% (m/m) in these SEDDS. SEDDS pre-concentrate (50  $\mu\text{l}$ ) was emulsified in 5 ml of 0.1 M PBS, pH 6.8 (1:100). Mean droplets size, polydispersity index (PI) and zeta potential of the formed emulsions were determined by using a Zeta sizer Nano-ZS (Malvern instruments, UK). All measurements were performed at room temperature.

**Table 1**  
Anionic surfactants used for hydrophobic ion pairing with trypsin.

Surfactant	Chemical group	Carbon atoms
 Sodium dodecylsulfate (SDS)	Sulfate	12
 Sodium taurocholate (ST)	Sulfonate	26
 Sodium deoxycholate (SDO)	Carboxylate	24

**Table 2**  
Compositions (% m/m), average droplet sizes and polydispersity index (PI) of the developed SEDDS formulations. Data shown as mean  $\pm$  SD (n = 3).

SEDDS	Captex 300	Kolliphor EL	PG	Droplet size (nm)	Trypsin complex	Zeta potential (mv)	PI
Blank	20	50	30	29.92 $\pm$ 0.09	–	–2.36	0.116
SDS	20	50	30	32.52 $\pm$ 0.14	1	–4.25	0.234
ST	20	50	30	39.15 $\pm$ 0.37	1	–4.06	0.265
SDO	20	50	30	30.49 $\pm$ 0.26	1	–3.38	0.181

### 2.2.6. Determination of enzymatic activity

Enzymatic activity of free trypsin, trypsin complexes and their corresponding SEDDS emulsions was determined using BAEE ( $N_\alpha$ -benzoyl-L-arginine ethyl ester) enzyme assay [16]. Briefly, BAEE solution (0.25 mM) in 67 mM sodium phosphate buffer, pH 7.6 at 25 °C was used as substrate. Test samples (trypsin solution, complex solutions and SEDDS emulsions) were prepared in 1 mM HCl with 20% DMSO. Concentrations of test samples were 0.5 mg/ml (trypsin), 1 mg/ml (SDS and ST complex/SEDDS) and 0.625 mg/ml (SDO complex/SEDDS). Each test sample was added to substrate solution at the volume ratio of 1:40 and after mixing by inversion, was immediately analyzed for increase in  $A_{253}$  on TECAN (Infinite M 200, Austria, GmbH), using one minute time intervals and a minimum of four data points. Enzymatic activity was calculated as BAEE units per ml of complex solution/ SEDDS emulsion using the following equation:

$$\text{BAEE units/ml enzyme} = \frac{(\Delta A_{253}/\text{minute Test} - \Delta A_{253}/\text{minute Blank})}{(0.001) \times (0.075)} \times \text{df}$$

where:

df = dilution factor

0.001 = change in  $A_{253}$ /minute based on unit definition

0.075 = volume (ml) of test sample used in assay

Results were expressed as percentage activity in comparison to enzymatic activity of free trypsin solution.

### 2.2.7. Interface analysis

In order to confirm the assembly of trypsin complexes on the surface of SEDDS droplets, zeta potential measurements of blank as well as each complex loaded SEDDS having been diluted 1:100 in water with and without 0.05% (m/v) of Bowman-Birk inhibitor (BBI) were performed. All formulations were incubated at 37 °C for 15 min prior to measurement.

### 2.2.8. Mucus collection and purification

Mucus was collected from the intestine of freshly slaughtered pigs using a method previously described by Nazir et al. [17]. The intestine was brought from local abattoir under ice cooling conditions to laboratory. Intestinal part containing chime was discarded. Remaining intestine was cut into longitudinal sections and mucus was collected by gently scraping intestinal surface with a microscopic slide. For purification, to each 1 g of mucus, 5 ml of 0.1 M sodium chloride solution was added and kept on gentle agitation (100 rpm) for 1 h at 4 °C followed by centrifugation for 2 h at 10,400g under cool conditions (10 °C). Supernatant was discarded

and the process was repeated once more. Purified mucus was stored at  $-20\text{ }^{\circ}\text{C}$  until use.

### 2.2.9. Mucus permeation studies

Mucus permeation studies were performed by using Transwell technique [18]. Fluorescein diacetate (FDA) was incorporated into blank SEDDS as well as complex loaded SEDDS up to 1% (m/m). For mucus permeation test, 24-well plates (Greiner-BioOne, Kremsmünster, Upper Austria, Austria) were used occupying a surface of  $33.6\text{ mm}^2$ . The acceptor compartment was filled with  $500\text{ }\mu\text{l}$  of 0.1 M PBS at pH 6.8. The surface of donor compartment was covered with 70 mg of purified mucus resulting in a 2.1 mm thick mucus layer before adding  $250\text{ }\mu\text{l}$  of each FDA labelled SEDDS formulation (1:100 in corresponding buffer). The plate was covered with the lid and kept on shaking board at 300 rpm (Vibramax 100; Heidolph Instruments, Schwabach, Bavaria, Germany) which was placed in an incubator at  $37\text{ }^{\circ}\text{C}$ . For positive control of each sample, donor compartments without mucus were used. For negative control,  $250\text{ }\mu\text{l}$  of 0.1 M PBS was added to the donor compartment containing mucus layer. Aliquots of  $100\text{ }\mu\text{l}$  were withdrawn from acceptor compartments at the time points of 0, 1, 2, 3 and 4 h and withdrawn volume was replaced at each time point by 0.1 M PBS pH 6.8 maintained at  $37\text{ }^{\circ}\text{C}$ . To each  $100\text{ }\mu\text{l}$  aliquot,  $20\text{ }\mu\text{l}$  of 5 M NaOH was added and the mixture was incubated at  $37\text{ }^{\circ}\text{C}$  for 30 min in order to completely hydrolyze FDA into sodium fluorescein. The resulting fluorescein was measured in 96-well plate using microplate reader of TECAN (Infinite M 200, Austria, GmbH) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Permeation of enzyme decorated SEDDS was calculated at each time point compared to blank SEDDS. The results were expressed as the percentage permeation quantified by the amount of FDA in the acceptor compartment.

### 2.2.10. Determination of diffusion coefficient (Log D)

In order to assess distribution of trypsin HIP decorated SEDDS, diffusion coefficient (log D) of free trypsin and each complex between SEDDS preconcentrate and 0.1 M PBS pH 6.8 as release

$$\text{Viable cells}(\%) = 100 - \left\{ \left( \frac{\text{Average experimental value} - \text{Negative control value}}{\text{Positive control value} - \text{Negative control value}} \right) \times 100 \right\}$$

medium was calculated [19]. For this, each substance was dissolved in respective SEDDS preconcentrate in different concentrations ranging from 0.1 mg to 3 mg. The maximum completely dissolved quantity of substance was considered as its solubility in SEDDS ( $S_{\text{SEDDS}}$ ). In order to determine solubility in release medium ( $S_{\text{RM}}$ ), each substance was dissolved in 0.1 M PBS pH 6.8 at 400 rpm,  $37\text{ }^{\circ}\text{C}$  for the period of 4 h. After this each sample was centrifuged and supernatant was analyzed using spectrophotometer to find out  $S_{\text{RM}}$ . Log D was calculated by using following equation:

$$\text{LogD} = \log \frac{S_{\text{SEDDS}}}{S_{\text{RM}}}$$

where

$S_{\text{SEDDS}}$  = solubility in SEDDS preconcentrate  
 $S_{\text{RM}}$  = solubility in the release medium

### 2.2.11. Rheological properties

Rheological studies were performed according to method previously described by our research group using a plate-plate combination rheometer (Haake Mars Rheometer, 379–0200, Thermo

Electron GmbH, Karlsruhe, Germany) with a frequency of 1 Hz at a temperature of  $37\text{ }^{\circ}\text{C}$  [20]. The gap between two plates was chosen about 0.5 mm. Each SEDDS nanoemulsion and trypsin solution (0.1% in 0.1 M PBS, pH 6.8) was mixed with purified intestinal mucus in the ratio of 1:2 (v/m) and equilibrated in the thermomixer at 300 rpm at  $37\text{ }^{\circ}\text{C}$ . Each sample was transferred to the rheometer for rheological measurement at different time points; 0, 15, 30, 60 and 120 min. The values were recorded in the shear stress range of 0.1–10 Pa. Each measurement was performed in triplicate.

### 2.2.12. Resazurin assay

Cytotoxic potential of each complex loaded SEDDS formulation was determined using resazurin assay according to a previously described method with some minor modifications [17]. Briefly human colorectal adenocarcinoma-derived cells (Caco-2) were seeded in a 24 well plate at a density of 25,000 cells per well in minimum essential medium (MEM) supplemented with penicillin/streptomycin solution (100 units/0.1 mg/L) and 10% (v/v) fetal calf serum (FCS). Cells were incubated for 14 days at  $37\text{ }^{\circ}\text{C}$  under 5%  $\text{CO}_2$  and 95% relative humidity environment. During incubation, medium was replaced after every 48 h. For assay, cells were washed twice with preheated 25 mM HEPES buffered saline (HBS) pH 7.4. Test solutions prepared in HBS at 3 different concentrations (0.5, 1 and 1.5% v/v), a negative control (HBS), and a positive control (1% v/v Triton X-100) were added in triplicate to the cell culture plate in 0.5 ml volume per well and incubated at  $37\text{ }^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  environment for 4 h. After incubation, test solutions were removed and the cells were washed with preheated HBS pH 7.4. An aliquot of  $250\text{ }\mu\text{l}$  of 2.2 mM resazurin solution was added to each well, and the cells were incubated again under the same conditions for 3 h. Afterwards, fluorescence of the supernatant from each well was measured at 540 nm excitation wavelength and 590 nm emission wavelength (TECAN Infinite M200, Austria GmbH).

Cell viability was calculated by following equation:

### 2.2.13. Statistical data analysis

Graph Pad Prism 5 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 for analysis of the results of proteolytic activity, mucus permeation and rheological studies. Independent student sample *t* test with  $P < 0.05$  was used to analyze the results of interface analysis.

## 3. Results and discussion

Trypsin decorated self-emulsifying drug delivery system was designed to overcome mucus permeation barrier. SEDDS formulations were characterized regarding droplet size, polydispersity index, zeta potential, enzymatic activity, mucus permeation, cytotoxicity and rheological properties.

### 3.1. Hydrophobic ion pairing (HIP)

HIP is a strategy which can be used to improve the lipophilicity of an ionizable hydrophilic molecule through simple electrostatic

interaction of a hydrophobic counter ion [11]. Hydrophobicity of trypsin was increased by HIP with anionic surfactants in order to facilitate its incorporation in SEDDS. Surfactants with different anionic moiety were selected for hydrophobic ion pairing. As trypsin has an isoelectric point of 10.3–10.7, a pronounced net positive charge can be induced in the acidic milieu [21]. Hydrophobic ion pair is formed by electrostatic interaction following by neutralization of positive charges in trypsin by oppositely charged surfactant molecules. Complex formation increased with increasing concentration of anionic surfactant until maximum complexation was achieved. Increasing the surfactant beyond this concentration did not further increase complexation and led in case of SDS even to a decrease in complexation efficiency.

The complexation efficiency of trypsin at different molar ratios of each surfactant is illustrated in Fig. 1. Among all tested trypsin/surfactant molar ratios, maximum complexation, 94.5%, was achieved in case of SDS at a ratio 1:5 and in case of ST, 85.7% at a ratio 1:8. In contrast, there was no significant difference of precipitated trypsin in case of all tested trypsin/SDO ratios. For further studies maximum trypsin surfactant complexation ratios of SDS and ST and in case of SDO, the 1:9 ratio were used.

In theory an equimolar ratio of negatively charged surfactant to positively charged amino acids should lead to maximum precipitation efficiency [22]. According to amino acid sequence studies of porcine trypsin, there are 7 net positive charges on the enzyme [23]. So the observed maximum precipitation ratios of 1:5, 1:8 and 1:9 for SDS, ST and SDO, respectively, seem to be close to the theoretical ratio of 1:7 for complete neutralization of charged moieties. Trypsin HIPs were obtained by ionic interactions between the enzyme and anionic surfactants under acidic pH conditions. In order to study the dissociation of complexes under different pH conditions, each complex was dissolved in water at pH 6.8, 7.2 and 8, at 37 °C. After 4 h, undissolved complexes were centrifuged and amount of dissociated trypsin from each complex was determined using spectrophotometer. At pH 6.8 and 7.2, trypsin dissociation was not more than 20%, whereas at pH 8, almost 30% trypsin dissociation was observed.

Log P value of trypsin was increased due to HIP as illustrated in Fig. 2. Ion pairing of trypsin with anionic surfactants was further confirmed by FTIR spectroscopy. Results of this study are shown in S1. IR spectra of trypsin complexes differ among each other and from that of original trypsin in the fingerprint region.

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jcis.2018.07.057>.

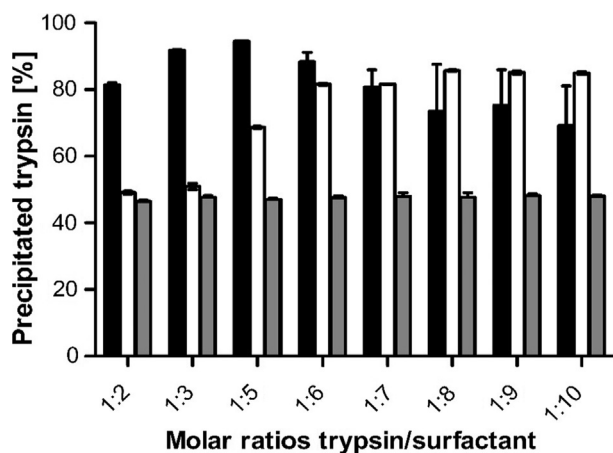


Fig. 1. Percentage precipitation of trypsin with SDO (grey bars), SDS (black bars) and ST (white bars) at different trypsin/surfactant molar ratios. Data is shown as mean  $\pm$  SD ( $n = 3$ ).

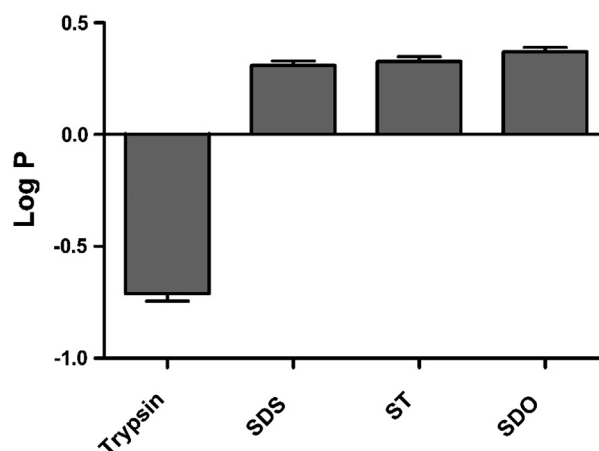


Fig. 2. Log P (octanol/water) of trypsin and its HIP complexes with SDS, ST and SDO. Indicated values are mean  $\pm$  SD ( $n = 3$ ).

### 3.2. Development and characterization of SEDDS

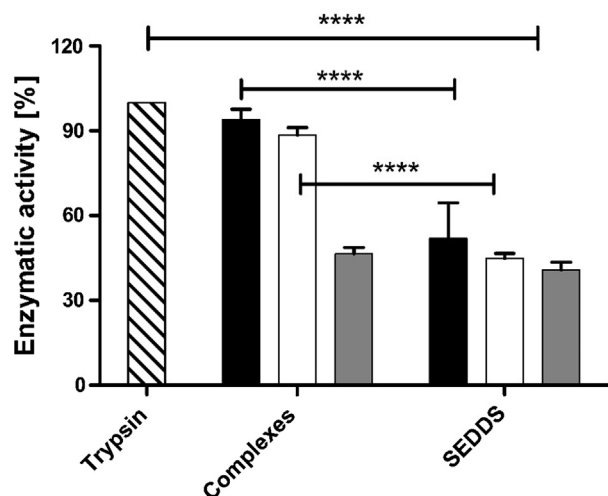
Designing of SEDDS require multiple components, such as oils, surfactants and co-surfactants. Various combinations of oil, surfactant and co-surfactants were characterized regarding homogeneity, emulsification time, phase separation and droplets size in order to obtain optimized formulations of SEDDS. Based on SEDDS properties, Captex 300, Kolliphor EL and propylene glycol were identified as suitable oil, surfactant and co-surfactants for SEDDS, respectively. Blank SEDDS as well as three complex containing formulations were prepared. Data regarding composition and properties of all SEDDS are shown in Table 2.

Visual appearance of SEDDS emulsion was evaluated by using the grading system. According to this system, emulsion having clear or slightly bluish appearance rapidly forming in <1 min corresponds to 'Grade A' [24]. Emulsification time of all SEDDS was found <1 min resulting in the rapid formation of clear nano-emulsions.

Complexes were loaded at a concentration of 1% in SEDDS for comparing their mucus permeation enhancing effect with that of SEDDS containing 1% papain HIP previously reported by our research group [11]. Droplets size of SEDDS was found in the range of 29.92–39.15 nm and PDI in the range of 0.116–0.265 as illustrated in Table 2. Additionally the zeta potential of SEDDS was found negative suggesting their comparatively higher mucus permeation ability due to less ionic interaction with negatively charged mucus compared to positively charged particles [25]. Zeta potential of blank SEDDS was decreased due to loading of each complex. Changes in droplet size and zeta potential of all SEDDS formulations is shown in Figs. S2 and S3.

### 3.3. Enzymatic activity

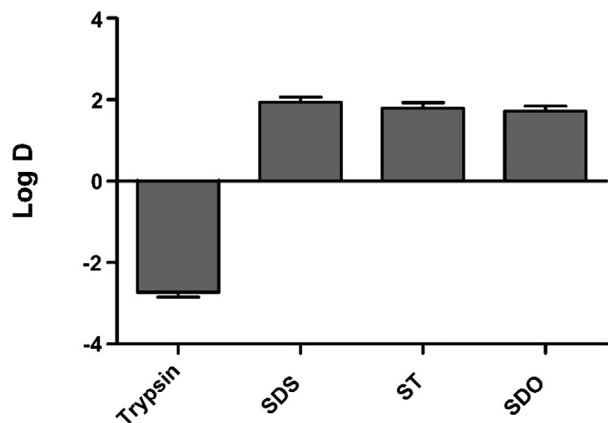
Proteolytic activity of free trypsin, complexes and SEDDS was determined by BAEE assay. In this study, quantity of each complex and SEDDS equivalent to 0.5 mg trypsin was used in the preparation of test samples to measure enzymatic activity. These quantities were calculated from trypsin: surfactant percentage ratios in each complex; 1:1 (SDS and ST) and 3:1 (SDO). Percentage enzymatic activity of trypsin in each complex and SEDDS were expressed in comparison to activity of free trypsin. Results of this study are illustrated in Fig. 3. Enzymatic activity of trypsin in complexes with SDO and SDS was 93.9% and 88.5%, respectively corresponding to activity of free trypsin. However, activity of trypsin in the complex with ST was 46.4%. In contrast, there was no signifi-



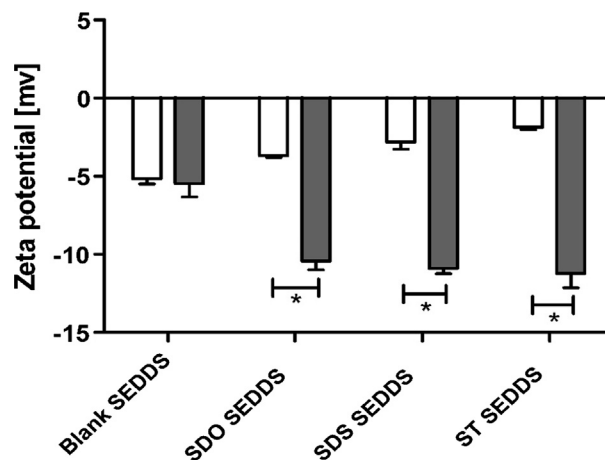
**Fig. 3.** Percentage of remaining enzymatic activity of trypsin in complexes and in SEDDS formulations; SDO (black bars), SDS (white bars) and ST (grey bars). Data is shown as mean  $\pm$  SD ( $n = 3$ ) (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ).

cant difference in enzymatic activity among SEDDS formulations. The enzymatic activity of trypsin complexed with SDO, SDS and ST in SEDDS was 51.9%, 44.8% and 40.7% respectively. As it was proven by a previous research study that proteases are not able to degrade peptides in the oily phase of SEDDS [26], proteolytic activity of SEDDS formulations might provide additional evidence for the assembly of trypsin HIPs on the interface of the droplets.

The preservation of enzymatic activity requires that on active site of enzyme, amino acid residues should remain unmodified. Amino acids of the active site of trypsin include His<sub>56</sub>, Asp<sub>90</sub>, Ser<sub>183</sub>, and Ser<sub>198</sub> [23]. As none of these amino acids bears a positive charge, in theory at least ionic interactions of anionic surfactants used can be excluded. However, attachment of surfactant molecules to cationic sites may cause bulkiness and hinder the substrate to access the active site. Less enzymatic activity of SEDDS compared to complexes might be explained by the hydrophilic environment being essential for its catalytic activity. Therefore, a higher affinity towards oily phase limits the free access of HIP to interact with the substrate in the aqueous phase. Log D values were determined to assess lipophilicity of trypsin HIP complexes. Data are shown in Fig. 4. Log D of complexes was found in the range of 1.6–2 at pH 6.8 suggesting moderate affinity towards oily droplets of SEDDS. In enzyme decorated SEDDS, complex is more likely to reside at the surface of oily droplets after SEDDS disper-



**Fig. 4.** Log D<sub>SEDDS/release medium</sub> of trypsin and trypsin complexes. Data is shown as mean  $\pm$  SD ( $n = 3$ ).

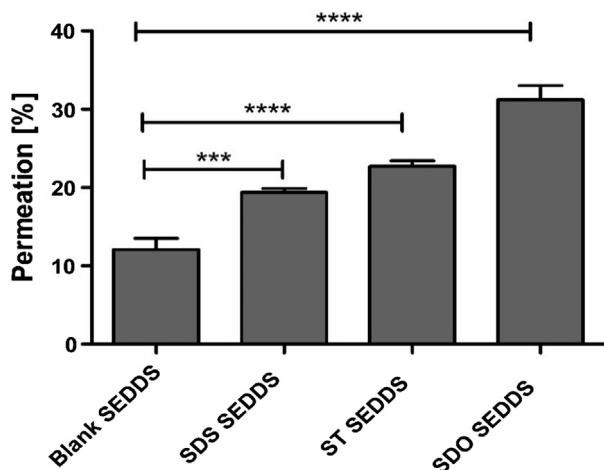


**Fig. 5.** Zeta potential of blank SEDDS and indicated HIP loaded SEDDS in water. Zeta potential, before addition of BBI (white bars), after addition of BBI (grey bars). Data is shown as mean  $\pm$  SD ( $n = 3$ ) (\* $P < 0.05$ ).

sion showing enzymatic activity. In order to further evaluate the location of complex in SEDDS, FITC labeled complexes were incorporated in SEDDS and fluorescence of SEDDS emulsions prepared using 0.1 M PBS pH 6.8 was measured at absorption wavelength 495 nm and emission wavelength of 520 nm. Fluorescent measurements are given in Fig. S4. All SEDDS exhibited more than 50% fluorescence activity as compare to the control solution of FITC prepared in the same concentration as in the SEDDS using 0.1 M PBS pH 6.8 as solvent. Presence of fluorescence detection in SEDDS emulsions suggested that the complex is not entirely located inside the oily core but resides at the oil/ dilution medium interface within the formed emulsions. Additionally SEDDS droplet interface was evaluated by measuring zeta potential. For this purpose, Bowman-Birk inhibitor (BBI) was used which is selectively binding to trypsin. It has an isoelectric point of pH 4.2 [27]. When trypsin is located on the interface of the oily droplets, BBI can bind to it causing a shift in zeta potential to more negative. Results of this study are illustrated in Fig. 5. Blank SEDDS with no trypsin at interface did not show statistically significant change ( $P < 0.05$ ) in zeta potential, whereas in case of each complex loaded SEDDS significant changes ( $P < 0.05$ ) in zeta potential were monitored after addition of BBI. Results suggest that within SEDDS trypsin HIPs are located at SEDDS droplet interface interacting with BBI and leading to alteration in surface charge.

#### 3.4. Mucus permeation studies

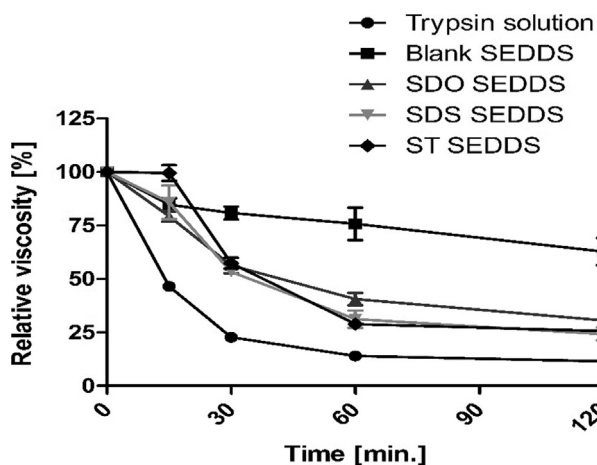
Mucus permeation properties of a drug delivery system are crucial to ensure an adequate level of drug in blood circulation [28]. In this study fluorescein diacetate (FDA) was used as a fluorescent marker to label the SEDDS. It is an uncharged lipophilic molecule with a molecular weight of 416 Da [29]. Use of FDA as permeation marker in SEDDS formulations has already been reported [11,24]. The membrane pore size of Transwell was 0.4  $\mu$ m which on the one hand minimizes the effect of membrane on permeation of SEDDS and on the other hand avoids migration of mucus to the acceptor compartment. Results of this study are illustrated in Fig. 6. The study was carried out for a period of 4 h as the intestinal residence time ranges from 4 to 6 h [30]. Each complex loaded SEDDS significantly increased the droplet permeation through mucus. Increase in mucus permeation was 2.6-fold for SDO SEDDS and 1.88- and 1.6-fold for ST and SDS SEDDS respectively in comparison to blank SEDDS. There was no significant difference among SDS and ST permeation whereas SDO showed a significant increase



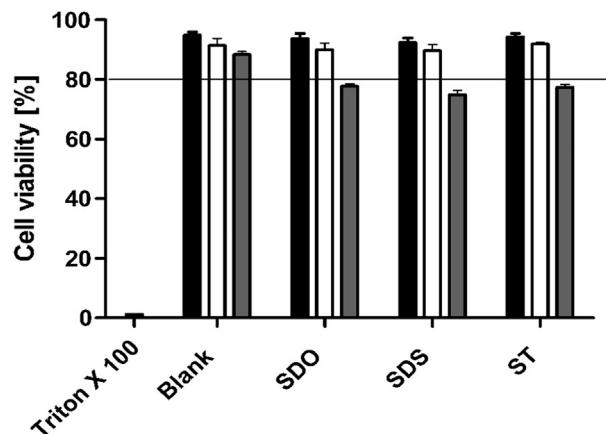
**Fig. 6.** Percentage permeation of blank and trypsin decorated SEDDS formulations using porcine intestinal mucus after 4 h at 37 °C. Data is shown as mean  $\pm$  SD (n = 3) (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001).

in mucus permeation as compare to other SEDDS. Factors which can affect the mucus permeation of enzyme decorated SEDDS are mucolytic activity, droplet size and zeta potential [31]. Blank SEDDS demonstrated significant lower mucus permeation than enzyme decorated SEDDS although their droplet size was comparable and zeta potential was negative. So it is assumed that mucus permeation enhancement properties of trypsin decorated SEDDS were based on mucolytic action of enzyme anchored.

Major determinant of integrity and viscoelastic properties of mucus is the glycoprotein constituent known as mucin which can form gel (mucus) upon aggregation. Glycoproteins in the gel matrix are linked by relatively weak non-covalent forces resulting in a flexible network [4]. It is recognized from earlier research studies that proteases cause interruption of mucus network structure resulting in leakier mucus with decreased viscosity [32]. In order to further evaluate the mucolytic effect of trypsin decorated SEDDS, rheological studies were performed. Results of this study are illustrated in Fig. 7. A significant decrease in dynamic viscosity of mucus overtime in case of enzyme decorated SEDDS shows mucolytic activity, whereas blank SEDDS did not show any significant effect on mucus viscosity. As compared to SEDDS, trypsin



**Fig. 7.** Rheological comparison of the mucolytic activity of trypsin solution, blank SEDDS, SDO SEDDS, SDS SEDDS and ST SEDDS. Mucus was incubated with trypsin solution and trypsin decorated SEDDS at 37 °C. Measurements were taken at indicated time intervals. Data is shown as mean  $\pm$  SD (n = 3).



**Fig. 8.** Cytotoxicity of blank SEDDS and complex loaded SEDDS at indicated concentrations; 0.5% (black bars), 1% (white bars) and 1.5% (grey bars), after 4 h incubation using resazurin assay. Data is shown as mean  $\pm$  SD (n = 3).

solution caused more rapid decline in mucus viscosity as compare to each trypsin decorated SEDDS. In solution, trypsin can easily access all peptide bonds of mucin. In SEDDS, trypsin HIP being anchored on the surface of oily droplets can access those peptide bonds that are in the nearby surroundings, more likely to exert local mucolytic action and not completely destroying the entire mucus network.

### 3.5. Cytotoxicity studies

As oils and surfactants have been reported to be harmful at higher concentrations due to interaction with cell membrane [33], cytotoxic potential of developed SEDDS formulations was evaluated using resazurin assay. Resazurin assay is based on the ability of viable cells to reduce resazurin into resorufin (pink/ fluorescent) which can be measured by fluorometer at an excitation wavelength of 540 nm and an emission wavelength of 590 nm [17]. Enterocytes are the main cells found in small intestine. Therefore, Caco-2 cells were used as these cells can form monolayers of enterocytes like cells used in intestinal absorption studies [34]. As depicted in Fig. 8, increasing concentration of blank SEDDS from 0.5% to 1.5% (v/v) did not reduce cell viability below 80%. All three complex loaded formulations showed comparable results and were toxic at 1.5% concentration with cell viability below 80%. Furthermore, detachment of cells monolayer from a few wells containing 1.5% SEDDS concentration was observed. It might be due to interaction of trypsin complexes at higher concentration with cell membrane. So trypsin decorated SEDDS formulations can be considered relatively safe up to 1% concentration.

## 4. Conclusions

We developed a novel mucus permeation enhancing drug delivery system by decorating SEDDS with hydrophobically modified trypsin as an attempt to overcome the major barrier “mucus” encountered by drugs administered through oral route. In contrast to chemical modifications that can alter the enzyme’s proteolytic properties [35], HIP approach was used for reversible trypsin modification. Trypsin decorated SEDDS exhibited 2.6-fold improved mucus permeating properties in comparison to blank SEDDS whereas previously reported papain loaded mucolytic SEDDS accelerated mucus permeation just up to 2-fold [11]. Furthermore, the use of trypsin as mucus permeation enhancer might be a safer alternative to papain and bromelain regarding immunogenicity being more likely associated with these exogenous enzymes.

Consequently this system can be considered as promising vehicle capable of carrying drugs across the mucus barrier resulting in higher bioavailability.

### Acknowledgements

The authors are thankful and would like to extend acknowledgement to Higher Education Commission of Pakistan (HEC) and the Austrian Agency for International Cooperation in Education and Research, Austria (ÖAD) for their support.

### References

- [1] M. Boegh, M. Garcia-Diaz, A. Mullertz, H.M. Nielsen, Steric and interactive barrier properties of intestinal mucus elucidated by particle diffusion and peptide permeation, *Eur. J. Pharm. Biopharm.* 95 (Pt A) (2015) 136–143.
- [2] A. Lechanteur, A. das Neves, A. Sarmento, The role of mucus in cell-based models used to screen mucosal drug delivery, *Adv. Drug Deliv. Rev.* 124 (2018) 50–63.
- [3] M. Boegh, H.M. Nielsen, Mucus as a barrier to drug delivery—understanding and mimicking the barrier properties, *Basic Clin. Pharmacol. Toxicol.* 116 (3) (2015) 179–186.
- [4] H.H. Sigurdsson, J. Kirch, C.-M. Lehr, Mucus as a barrier to lipophilic drugs, *Int. J. Pharm.* 453 (1) (2013) 56–64.
- [5] A. Bernkop-Schnürch, R. Fragner, Investigations into the diffusion behavior of polypeptides in native intestinal mucus with regard to their peroral administration, *Pharm. Pharmacol. Commun.* 2 (8) (1996) 361–363.
- [6] L. Inchaurreaga, N. Martin-Arbella, V. Zabaleta, G. Quincoces, I. Penuelas, J.M. Irache, In vivo study of the mucus-permeating properties of PEG-coated nanoparticles following oral administration, *Eur. J. Pharm. Biopharm.* 97 (Pt A) (2015) 280–289.
- [7] I.P. de Sousa, C. Steiner, M. Schmutzler, M.D. Wilcox, G.J. Veldhuis, J.P. Pearson, et al., Mucus permeating carriers: formulation and characterization of highly densely charged nanoparticles, *Eur. J. Pharm. Biopharm.* 97 (2015) 273–279.
- [8] C. Menzel, A. Bernkop-Schnürch, Enzyme decorated drug carriers: targeted swords to cleave and overcome the mucus barrier, *Adv. Drug Deliv. Rev.* 124 (2018) 164–174.
- [9] T. Vasconcelos, S. Marques, B. Sarmento, Measuring the emulsification dynamics and stability of self-emulsifying drug delivery systems, *Eur. J. Pharm. Biopharm.* 123 (2018) 1–8.
- [10] N. Anton, T.F. Vandamme, Nano-emulsions and micro-emulsions: clarifications of the critical differences, *Pharm. Res.* 28 (5) (2011) 978–985.
- [11] C. Lechner, C. Menzel, F. Laffleur, A. Bernkop-Schnürch, Development and in vitro characterization of a papain loaded mucolytic self-emulsifying drug delivery system (SEDDS), *Int. J. Pharm.* 530 (1–2) (2017) 346–353.
- [12] A. Glazer, E.L. Smith, *The Enzymes*, Elsevier Inc, 1971, pp. 501–546.
- [13] C.R. Limber, H.G. Reiser, L.C. Roettig, G.M. Curtis, Enzymatic lysis of respiratory secretions by aerosol trypsin, *J. Am. Med. Assoc.* 149 (9) (1952) 816–821.
- [14] S.M. Farber, S.L. Pharr, D.A. Wood, R.D. Gorman, The mucolytic and digestive action of trypsin in the preparation of sputum for cytologic study, *Science* 117 (1953) 687–690.
- [15] J. Griesser, G. Hetényi, M. Moser, F. Demarne, V. Jannin, A. Bernkop-Schnürch, Hydrophobic ion pairing: key to highly payloaded self-emulsifying peptide drug delivery systems, *Int. J. Pharm.* 520 (1) (2017) 267–274.
- [16] H.-U. Bergmeyer, *Methods of Enzymatic Analysis*, Academic Press, New York, 1974.
- [17] I. Nazir, C. Lechner, B. Le-Vinh, A. Bernkop-Schnürch, Surface phosphorylation of nanoparticles by hexokinase: a powerful tool for cellular uptake improvement, *J. Colloid Interface Sci.* 516 (2018) 384–391.
- [18] H. Friedl, S. Dünnhaupt, F. Hintzen, C. Waldner, S. Parikh, J.P. Pearson, et al., Development and evaluation of a novel mucus diffusion test system approved by self-nanoemulsifying drug delivery systems, *J. Pharm. Sci.* 102 (12) (2013) 4406–4413.
- [19] A. Bernkop-Schnürch, A. Jalil, Do drug release studies from SEDDS make any sense?, *J. Control. Release* 271 (2018) 55–59.
- [20] D. Sakloetsakun, J.M. Hombach, A. Bernkop-Schnürch, In situ gelling properties of chitosan-thioglycolic acid conjugate in the presence of oxidizing agents, *Biomaterials* 30 (31) (2009) 6151–6157.
- [21] P. Voytek, E.C. Gjessing, Studies of an anionic trypsinogen and its active enzyme from porcine pancreas, *J. Biol. Chem.* 246 (2) (1971) 508–516.
- [22] E.L. Smith, J. Kimmel, D.M. Brown, Crystalline papain II. Physical studies; the mercury complex, *J. Biol. Chem.* 207 (2) (1954) 533–549.
- [23] M.A. Hermodson, L.H. Ericsson, H. Neurath, K.A. Walsh, Determination of the amino acid sequence of porcine trypsin by sequenator analysis, *Biochemistry* 12 (17) (1973) 3146–3153.
- [24] O. Zupančič, J.A. Griefinger, J. Rohrer, I.P. de Sousa, L. Danninger, A. Partenhauser, et al., Development, in vitro and in vivo evaluation of a self-emulsifying drug delivery system (SEDDS) for oral enoxaparin administration, *Eur. J. Pharm. Biopharm.* 109 (2016) 113–121.
- [25] J.S. Crater, R.L. Carrier, Barrier properties of gastrointestinal mucus to nanoparticle transport, *Macromol. Biosci.* 10 (12) (2010) 1473–1483.
- [26] G. Hetényi, J. Griesser, M. Moser, F. Demarne, V. Jannin, A. Bernkop-Schnürch, Comparison of the protective effect of self-emulsifying peptide drug delivery systems towards intestinal proteases and glutathione, *Int. J. Pharm.* 523 (1) (2017) 357–365.
- [27] X. Li, Y. Hua, Y. Chen, X. Kong, C. Zhang, Two-step complex behavior between Bowman-Birk protease inhibitor and ι-carrageenan: effect of protein concentration, ionic strength and temperature, *Food Hydrocolloids* 62 (2017) 1–9.
- [28] T. Iglesias, A. Lopez de Cerain, J.M. Irache, N. Martin-Arbella, M. Wilcox, J. Pearson, et al., Evaluation of the cytotoxicity, genotoxicity and mucus permeation capacity of several surface modified poly(anhydride) nanoparticles designed for oral drug delivery, *Int. J. Pharm.* 517 (1–2) (2017) 67–79.
- [29] O. Zupancic, A. Partenhauser, H.T. Lam, J. Rohrer, A. Bernkop-Schnürch, Development and in vitro characterization of an oral self-emulsifying delivery system for daptomycin, *Eur. J. Pharm. Sci.* 81 (2016) 129–136.
- [30] I.P. de Sousa, T. Moser, C. Steiner, B. Fichtl, A. Bernkop-Schnürch, Insulin loaded mucus permeating nanoparticles: addressing the surface characteristics as feature to improve mucus permeation, *Int. J. Pharm.* 500 (1) (2016) 236–244.
- [31] N.A. Efiara, T.N.Q. Phan, A.J. Wicaksono, A. Bernkop-Schnürch, Mucus permeating self-emulsifying drug delivery systems (SEDDS): about the impact of mucolytic enzymes, *Colloids Surf. Biointerfaces* 161 (2018) 228–235.
- [32] A. Mahmood, F. Laffleur, G. Leonaviciute, A. Bernkop-Schnürch, Protease-functionalized mucus penetrating microparticles: in-vivo evidence for their potential, *Int. J. Pharm.* 532 (1) (2017) 177–184.
- [33] A. Palamakula, M.A. Khan, Evaluation of cytotoxicity of oils used in coenzyme Q10 Self-emulsifying Drug Delivery Systems (SEDDS), *Int. J. Pharm.* 273 (1–2) (2004) 63–73.
- [34] J.Y. Lock, T.L. Carlson, R.L. Carrier, Mucus models to evaluate the diffusion of drugs and particles, *Adv. Drug Deliv. Rev.* 124 (2018) 34–49.
- [35] J. Stolarow, M. Heinzelmann, W. Yeremchuk, C. Syldatk, R. Hausmann, Immobilization of trypsin in organic and aqueous media for enzymatic peptide synthesis and hydrolysis reactions, *BMC Biotech.* 15 (2015) 77.