European Journal of Pharmaceutics and Biopharmaceutics 142 (2019) 92–100

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Imine bond formation: A novel concept to incorporate peptide drugs in self-emulsifying drug delivery systems (SEDDS)

Aida Dizdarević[a], Nuri Ari Efiana[a,b], Thi Nhu Quynh Phan[a,c], Barbara Matuszczak[d], Andreas Bernkop-Schnürch[a,*]

[a] Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innrain 80/82, A-6020 Innsbruck, Austria
[b] Department of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Ahmad Dahlan, Jl. Prof. Dr. Soepomo, S.H., Janturan, Warungboto, Umbulharjo, Yogyakarta 55164, Indonesia
[c] Faculty of Pharmacy, University of Medicine and Pharmacy, Hue University, 06 Ngo Quyen Street, Vinh Ninh Ward, Hue City, Thua Thien Hue Province 530000, Viet Nam
[d] Department of Pharmaceutical Chemistry, Institute of Pharmacy, University of Innsbruck, Innrain 80/82, A-6020 Innsbruck, Austria

ARTICLE INFO

Keywords:
Oral drug delivery
Nanocarriers
Polymyxin B
Cinnamaldehyde
Bovine serum albumin
SEDDS
Self-emulsifying drug delivery systems

ABSTRACT

Hypothesis: Because of its hydrophilic character the peptide drug Polymyxin B (PMB) cannot be incorporated in lipophilic nanocarrier systems such as self-emulsifying drug delivery systems (SEDDS) for oral administration. Due to the formation of imine conjugates between the primary amino groups of PMB and the carbonyl group of cinnamaldehyde, however, drug lipophilicity might be sufficiently raised for incorporation in SEDDS.
Methods: Imine bonds were formed between the primary amino groups of PMB and the carbonyl group of cinnamaldehyde. PMB-cinnamaldehyde conjugate was characterized regarding degree of substitution, log P and release of PMB due to interaction with bovine serum albumin (BSA), SEDDS loading and cell viability.
Results: 87.1% of primary amines formed imines with cinnamaldehyde. Log P was increased 69.183 – folds. BSA triggered release of PMB was 45.2%, 64.9% and 80.6% within 16 h. Log $D_{SEDDS/Release\ medium}$ of PMB-cinnamaldehyde conjugate was 3.4.
Conclusion: According to these findings, the concept of imine bond formation with cinnamaldehyde can be considered as a novel concept for increasing lipophilicity of the hydrophilic antibiotic peptide PMB.

1. Introduction

Within recent years self-emulsifying drug delivery systems (SEDDS) experience a renaissance as mucosal dosage forms, since it could be demonstrated in various studies that even hydrophilic macromolecular drugs can be incorporated in their lipophilic phase. SEDDS are isotropic mixtures of oils, surfactants and co-solvents forming fine oil-in water (O/W) emulsions with a typical droplet size of 100–300 nm when being introduced in an aqueous phase upon mild agitation [1]. Due to a hydrophobic ion pairing, highly hydrophilic peptide drugs such as exenatide [2] and octreotide [3], heparins such as enoxaparin [4] and even pDNA [5] can be incorporated in the SEDDS preconcentrate. Although these ion pairs are highly stable in the lipophilic phase of oily droplets, most of them are unstable in the aqueous phase of body fluids. This leads to an unintended fast release from the oily delivery system as the drug is to a much higher extent soluble in the aqueous release medium when the ion pair falls apart [6,7]. In addition a more stable lipophilic drug conjugate might be advantageous regarding membrane permeability and systemic uptake. Alternatives to hydrophobic ion pairing might therefore be a promising strategy to further advance the SEDDS technology. Hence, it was the aim of this study to evaluate the potential of another method to form lipophilic conjugates with hydrophilic drugs. Our concept is based on the formation of imine bonds between primary amine bearing drugs and lipophilic aldehydes that are generally regarded as safe. Such imine bonds as illustrated in the graphical abstract - known as Schiffs bases - are more stable than ionic bonds but likely still sufficiently unstable to fall apart in the systemic circulation. Generally, two different approaches can be used for imine formation [8,9]. Organic based imine formation involves reflux of an amine and an aldehyde in hazardous and volatile solvents such as methanol. On the other hand, water based imine formation is an environmental friendly alternative. In addition, it is a cheap procedure requiring no harsh conditions for synthesis. Although imine formation in organic solvents provides more stable products, it requires higher

* Corresponding author.
E-mail address: andreas.bernkop@uibk.ac.at (A. Bernkop-Schnürch).

https://doi.org/10.1016/j.ejpb.2019.06.002
Received 21 February 2019; Received in revised form 1 June 2019; Accepted 5 June 2019
Available online 06 June 2019
0939-6411/

reaction temperatures, that might cause denaturation in case of peptide drugs. Based on these considerations, an aqueous medium was chosen for the reaction [10]. Within this study polymyxin B (PMB) was selected as model peptide drug. Polymyxin B (PMB) is an antibiotic derived from the bacterium *Bacillus polymyxa* and used to treat a variety of infectious diseases caused by gram – negative bacteria such as *Pseudomonas aeruginosa* [11]. The drug has for instance been investigated for the treatment of cystic fibrosis when applied as combined therapy to patients with lung infections caused by gram – negative bacteria [12,13]. Because of its low oral bioavailability, however, only intravenous formulations are available on the market. For the formation of lipophilic conjugates the lipophilic aldehyde cinnamaldehyde was chosen due to its low toxicity and high lipophilic character with a log P value of 1.9 (pubchem). The conjugates were prepared in aqueous solution in different molar ratios. Since serum albumin is the most abundant plasma protein with a molecular weight of 66.3–66.9 kDa playing a key role in drug binding and transport, the potential of release of free PMB from PMB-cinnamaldehyde conjugates via exchange reactions with BSA was investigated [8,14,15,16]. In addition, PMB-cinnamaldehyde conjugates were incorporated in SEDDS and characterized regarding droplet size, polydispersity index, cytotoxicity and log $D_{SEDDS/RM}$.

2. Materials and methods

2.1. Materials

Polymyxin B sulphate (PMB), trans-cinnamaldehyde 99%, bovine serum albumin (BSA), trifluoracetic acid (TFA), sodium hydroxide (NaOH), Kolliphor EL, Tween 20 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Handels GmbH, Vienna, Austria. 1-Octanol-emplura was purchased from Merck, Darmstadt, Germany. Propylenglycol was purchased from Gatt-Koller, Absam, Innsbruck, Austria. Acetonitrile and purified water were purchased from VWR, Vienna, Austria. Capmul MCM was generously provided from Abitec, Barenz GmbH, Oberhausen Germany. Peceol was a gift from Gattefossé, Saint-Priest, France. All other chemicals were of analytical grade, pharmaceutical or food grade and were received from commercial vendors.

2.2. Methods

2.2.1. Quantification of polymyxin B and bovine serum albumin via HPLC

Polymyxin B sulphate (PMB) and bovine serum albumin (BSA) were quantified via HPLC system, Hitachi Elite LaChrom equipped with L-2130 pump, L-2200 autosampler and L2450 photodiode array UV detector. As a detection technique, reversed phase chromatography was employed by using Nucleosil 100-5 C 18 as a stationary phase. In order to provide effective separation of PMB isocratic and gradient HPLC methods as previously described for colistin were used [17]. As eluent A water was used and as eluent B acetonitrile (ACN) with 0.05% (v/v) trifluoroacetic acid (TFA). Isocratic method (35% mobile phase A and 65% mobile phase B) at 30 °C with a flow rate of 1.0 ml/min over 10 min was applied. Gradient method was used for detection of PMB in the partitioning coefficient (log P) investigations and for all other experiments isocratic method was used. PMB was detected at 35 °C with gradient elution and a flow rate of 1 ml/min: 0–5 min: from 90% A/ 10% B to 80% A/20% B; 5–7 min: 80% A/20% B to 35% A/65% B; 7–14 min: 35% A/65% B to 75% A/ 25% B; 14–15 min: 75% A/25% B to 80% A/ 20% B; 15–16 min: 80% A/ 20% B to 90% A/ 10% B (eluent A: water; eluent B: 0.05% (v/v) trifluoroacetic acid in acetonitrile) with signal determination by UV detector at 212 nm. For detection of BSA, isocratic method was employed and linearity of developed methods was confirmed. Calibration curves of PMB and BSA were generated at 212 nm in a range of 62.5–1000 µg/ml and confirmed as linear for both (poly)petides.

Before injection, eventually presented particles were removed by employing centrifugal force at 13.400 rpm for 5 min. Injection volume of each sample was 10 µl.

2.2.2. Preparation and characterization of PMB-cinnamaldehyde conjugate

In order to increase hydrophobicity of PMB, it was conjugated to cinnamaldehyde. For this purpose, PMB was dissolved in demineralized water in a final concentration of 1 mg/ml. Subsequently, cinnamaldehyde was added in different molar ratios from 2:1 to 1:10. After that, pH of the solutions was adjusted to 10.5 with 1 M NaOH followed by shaking in a thermomixer (Eppendorf Thermo Mixer® C, Hamburg, Germany) at 400 rpm and 25 °C for 1 h. The precipitated PMB-cinnamaldehyde conjugates were separated via centrifugation at 13.400 rpm for 10 min (MiniSpin®, Eppendorf Austria GmbH), lyophilized (Christ Gamma 1–16 LSC Freezedryer) and stored at 4 °C until further use. Concentration of free PMB in supernatants before and after conjugation was quantified via HPLC as described above.

Precipitation efficiency was calculated by using the following equation [18]:

$$precipitation\ \ efficiency\ \ (\%) = 1 - \frac{Concentration\ \ of\ \ free\ \ polymyxin\ \ B\ \ in\ \ supernatant\ \ after\ \ conjugation}{Concentration\ \ of\ \ free\ \ polymyxin\ \ B\ \ in\ \ supernatant\ \ before\ \ conjugation} \times 100 \quad (1)$$

In order to confirm imine bond formation, NMR and IR spectroscopy were employed. IR spectra were recorded on a Bruker ALPHA FT-IR apparatus equipped with a Platinum ATR (attenuated total reflection) module. The ^{1}H NMR spectra were recorded in DMSO-d_6 + D_2O solution in 5 mm tubes at 30 °C on a Varian Gemini 200 spectrometer (199.98 MHz). The centre of the solvent multiplet (DMSO-d_6) was used as internal standard (chemical shifts in δ ppm), which was related to tetramethylsilane (TMS) with δ 2.49 ppm.

2.2.3. Partitioning coefficient log P

Lipophilicity of PMB-cinnamaldehyde conjugates was characterized by Log P determinations. In brief, n-octanol and water were firstly saturated by mixing each of them in equal volumes of 5 ml and shaking in an Eppendorf thermomixer at 500 rpm and room temperature for 24 h. Further, the two phases were separated and water-saturated n-octanol was added in a volume of 500 µl to 1 mg of each formed PMB-cinnamaldehyde conjugate. The dispersions were vortexed and sonicated for 5 min followed by addition of 500 µl of n-octanol-saturated water. In parallel, a control experiment was conducted by dissolving 1 mg of PMB in 500 µl of n-octanol-saturated water and addition of 500 µl water-saturated n-octanol. The n-octanol/water phases containing PMB-cinnamaldehyde conjugates or PMB were kept shaking at 500 rpm and 37 °C for 24 h. The complete phase separation of octanol and water was achieved by centrifugation (13.400 rpm, 10 min). To determine the amount of PMB-cinnamaldehyde conjugates in octanol phase, prior to injection 10 µl of each octanol sample was diluted with methanol in a ratio 1–4 (v/v). In contrast, samples having been withdrawn from the water phase were injected without any dilution [18].

The conjugates were quantified in DAD at wavelength of 328 nm by isocratic method as described above and Log P was calculated as follows:

$$LogP = log_{10} \frac{Concentration\ \ of\ \ PMB-cinnamaldehyde\ \ conjugate\ \ in\ \ octanol\ \ phase}{Concentration\ \ of\ \ PMB-cinnamaldehyde\ \ in\ \ water\ \ phase} \quad (2)$$

Log P of free PMB was determined for comparison reasons by gradient method at 212 nm and calculated as described above.

2.2.4. Determination of degree of substitution by 2,4,6-trinitrobenzene sulfonic (TNBS) test

Free PMB as well as PMB-cinnamaldehyde conjugates formed in different molar ratios were characterized regarding free amino groups content and degree of substitution by using TNBS assay (Table 1). To

Table 1
Free amine content and degree of substitution of free amino groups with imines in conjugates calculated from 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay.

Conjugates formed in molar ratios PMB: cinnamaldehyde	Degree of substitution of free amino groups with imines (%)
2: 1	25.7
1: 3	68.2
1: 5	78.6
1: 8	80.2
1: 10	87.1

0.5 mg of unmodified PMB and PMB-cinnamaldehyde 0.500 ml of DMSO was added. Samples were then diluted in 100 mM borate buffered saline (BBS) pH 10 in range of 0.015–0.125 mg/ml. Further, 0.150 ml of previously diluted 500 - fold TNBS reagent in BBS was added to 0.300 ml of each previously diluted sample of PMB or PMB-cinnamaldehyde conjugate in DMSO/BBS. The samples were covered by aluminium foil and incubated at 37 °C in water bath for 2 h. Thereafter, samples were centrifuged (13.400 rpm, 5 min) and the absorbance was measured at 420 nm [19]. According to previously published study, free amine content was calculated by dividing the linear regression slope of each PMB-cinnamaldehyde conjugate with the slope of unmodified PMB [20]. Furthermore, the degree of substitution for each conjugate (DS) was calculated as follows:

$$DS = (1 - \text{free amino group}) \times 100\% \quad (3)$$

2.2.5. Release of free PMB from PMB-cinnamaldehyde conjugate

The release of PMB from PMB-cinnamaldehyde conjugate was evaluated in the presence of serum albumin. Therefore, 1 mg of PMB-cinnamaldehyde conjugate formed in ratio 1:5 was incubated in 1 ml of 4% (m/v) BSA solution while shaking at 37 °C for 16 h. For this purpose, pH of the medium was adjusted to 6, 6.5 and 7 with either 1 M NaOH or 1 M HCl. At predetermined time points, aliquots of 50 µl were withdrawn from the solutions while shaking and replaced with an equal volume of water followed by adjusting pH as described above. After centrifugation at 13.400 rpm for 5 min, samples were diluted in a ratio of 1–4 with demineralized water. PMB having been released from PMB-cinnamaldehyde conjugate was quantified via HPLC utilizing a standard curve.

2.2.6. Solubility studies and SEDDS development

SEDDS were developed based on our experience gained from previous studies utilizing various oils, surfactants and co-solvents. PMB-cinnamaldehyde conjugates were dispersed in organic solvents as listed in Table 2, stirred for 5 min and centrifugated at 13.400 rpm for 5 min. Complete dissolution of PMB-cinnamaldehyde conjugate in listed organic solvents was confirmed visually. Based on results of these solubility studies (Table 2) and those gained from previous studies, four blank SEDDS formulations A-D were developed (Table 3). Capmul MCM and Peceol as oils, Cremophor EL and Tween 20 as surfactants and

Table 2
Solubility studies of conjugate in different organic solvents, surfactants and co-solvents.

Solvents	Chemical name	HLB	Solubility
Captex 300	Caprylic/Capric triglyceride	1	No
Peceol	Glyceryl monooleate	1	Yes
Captex 200	Propylene glycol dicaprylate	NA	No
Capmul MCM EP	Glycerol monocaprylocaprate Type I	4.50	Yes
Cremophor EL	Macrogolglycerol ricinoleate	13	Yes
Capryol 90	Propylene glycol monocaprylate Type II	15	No
Tween 20	Polyoxyethylene sorbitan monolaurate	16.7	Yes
Transcutol	Diethylene glycol monoethyl ether	NA	Yes
Propylenglycol	Propane-1,2-diol	NA	Yes

Table 3
Composition and characterization of blank and loaded SEDDS in 1:100 phosphate buffered saline (PBS) at 37 °C for 0 h and 4 h. Data are shown as mean ± SD (n = 3).

	HLB	Capmul MCM	Peceol	Cremophor EL	Tween 20	Propylenglycol	Transcutol	Size (nm) of unloaded formulation at 0 h, Means	PDI of unloaded formulation at 0 h, means	Size (nm) of loaded formulation at 0 h/ 4h, means	PDI of loaded formulation at 0 h/4 h, means
A	10.3	25%	/	45%	/	30%	/	118.1 ± 1.1	0.317 ± 0.007	/	/
B	12.9	25%	/	/	45%	/	30%	166.2 ± 54.8	0.442 ± 0.03	/	/
C	9.6	/	34.6%	25%	30%	10.35%	/	506.56 ± 145.8	0.664 ± 0.2	/	/
D	11.2	22%	6.04%	18.9%	27.2%	25.8%	/	154.4 ± 1.2	0.388 ± 0.009	199.2 ± 3.3/214.1 ± 1.1	0.337 ± 0.04/0.295 ± 0.02

propylenglycol and Transcutol as co-solvents were therefore homogenized by using vortex and thermomixer at 900 rpm and short sonication [4]. To investigate the stability, SEDDS formulations were stored at room temperature for 24 h. Finally, SEDDS pre-concentrates were centrifuged at 13.400 rpm for 5 min confirming no phase separation. The nanoemulsions were prepared by diluting drug loaded/blank SEDDS in a ratio 1:100 in 20 mM phosphate buffer saline (PBS) pH 6.8. Mean droplet size as well as polydispersity index were determined by Nano-ZSP (Malvern instruments, Worcestershire, UK) at 37 °C directly after preparation and after 4 h. The measurements were executed using following set up: viscosity (0.6864 cP), refractive index (1.330), cell (disposable cuvettes-DTS0012), measurement angle (173° backscatter), data processing analysis model (normal resolution), software (Zetasizer Nano ZSP Malvern) and wavelength (4 mV; 637.8 nm).

2.2.7. Log D

To predict in vivo drug release from SEDDS the distribution coefficient (log $D_{SEDDS/RM}$) between SEDDS and release medium was determined [7]. For that purpose, the maximum concentration of PMB-cinnamaldehyde conjugate that can be dissolved in SEDDS as well as in the release medium was determined. On the one hand, 7 mg of PMB-cinnamaldehyde conjugate was dispersed in 100 µl of SEDDS pre-concentrate formulation D. On the other hand, 1 mg of PMB-cinnamaldehyde conjugate was dispersed in 1 ml of demineralized water used as release medium. Both dispersions were stirred for 12 h. Thereafter, the remaining undissolved PMB-cinnamaldehyde conjugate was removed by centrifugation. The supernatant of SEDDS pre-concentrate formulation D was diluted 1:20 with methanol, whereas the supernatant of the release medium was diluted 1:2 with 0.05% TFA in ACN prior to HPLC analyses. Samples were injected in HPLC in volume of 40 µl and quantified as described above. Log D was calculated by utilizing the following equation:

$$LogD = log_{10} \frac{Concentration\ \ of\ \ PMB-cinnamaldehyde\ \ conjugate\ \ in\ \ SEDDS}{Concentration\ \ of\ \ PMB-cinnamaldehyde\ \ conjugate\ \ in\ \ Release\ \ medium} \quad (4)$$

2.2.8. Cell viability

Cell viability experiments of SEDDS formulations were performed on Caco-2-cells by using Resazurin assay [21]. Caco 2-cells were supplied from European collection of cell cultures (ECACC, Health Protection Agency, Proton Down, Salisbury, Wiltshire, UK). The passage numbers of 35–40 were used. Approximately 2.5×10^4 cells per well were seeded to a 24-well plate. Cells were incubated in red MEM supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin solution (100 units/0.1 mg) at 95% humidity, 37 °C and atmosphere of 5% CO_2 for 14 days. On the day of experiment, Caco-2 cells were washed with pre-warmed Hank's balanced salt solution (HBSS) and incubated at 95% humidity, 37 °C and atmosphere of 5% CO_2 for 30 min. 0.1% (m/v) SEDDS in HBSS containing 3.6% PMB-cinnamaldehyde conjugate as well as 0.0036% m/v PMB-cinnamaldehyde conjugate and 0.0036% of free PMB dispersed in 2% DMSO/HBSS solution were added (500 µl/well) and the cells were incubated at 37 °C for 4 h. MEM without phenol red and 0.1% Triton X-100 in HBSS were used as negative and positive control, respectively. After incubation, SEDDS dispersions were removed. Cells were washed with HBSS followed by the addition of 250 µl of 5% resazurin dye in PBS pH 6.8. Cells were incubated for additional 3 h before undergoing fluorescent intensity measurement (Tecan infinite® M200 spectrophotometer, Austria). The measurement was performed at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Cell viability was calculated as follows:

$$Cell\ \ viability\ \ (\%) = \frac{As}{Ac} \times 100 \quad (5)$$

where As = fluorescent intensity of SEDDS dispersion, Ac = fluorescent intensity of negative control.

2.2.9. Statistical data analysis

Statistical data analysis was performed by using SPSS software. One way ANOVA tests with $p < 0.05$ as the minimal level of significance followed by Turkeýs multiple comparison test with $p < 0.05$ as minimal level of significance were performed. All experiments were performed in triplicate and the values are expressed as the means ± SD.

3. Results

3.1. Preparation of PMB-cinnamaldehyde conjugate

PMB possesses highly hydrophilic features because of five primary amino groups causing a cationic net charge (Chemicalize.org). In order to be incorporated in SEDDS, it was conjugated with a lipophilic aldehyde as illustrated in Fig. 1. After addition of cinnamaldehyde in different molar ratios to PMB aqueous solution (Fig. 2), precipitation occurred at pH ≥ 8 indicating the formation of imine bonds already under slightly alkaline conditions. Almost 100% precipitation efficiency was observed at molar ratios between 1:3 and 1:10 (Fig. 2). In contrast, precipitation efficiency at ratio of 2:1 was comparatively lower than that obtained at ratios of 1: ≥3. PMB is a basic peptide with p*K*a values of 9.07, 9.54, 9.84, 10.02 and 10.24 and an isoelectric point of 10.74 (Chemicalize.org). The net charge of polymyxin B was decreased by dropwise addition of 1 M NaOH. In the presence of cinnamaldehyde turbidity increased with increasing pH, whereas without aldehyde PMB solutions remained clear. According to this, only uncharged amines seem to react with aldehydes forming imine conjugates. Imine bond formation between PMB and cinnamaldehyde was additionally confirmed via IR and NMR. Comparison of the IR spectrum of the PMB-cinnamaldehyde conjugate with the unmodified PMB showed significant differences in the fingerprint region and confirmed the conjugation. At 1100 cm^{-1} there is no band in the spectrum of the PMB-cinnamaldehyde conjugate (Fig. 3). In the aliphatic part, the ^1H NMR spectrum of the polymyxin cinnamaldehyde derivative showed similar signals as observed in the spectrum of polymyxin. Additional signals between 6.6 and 8.2 ppm were attributed to the aromatic protons of the cinnamaldehyde subunit.

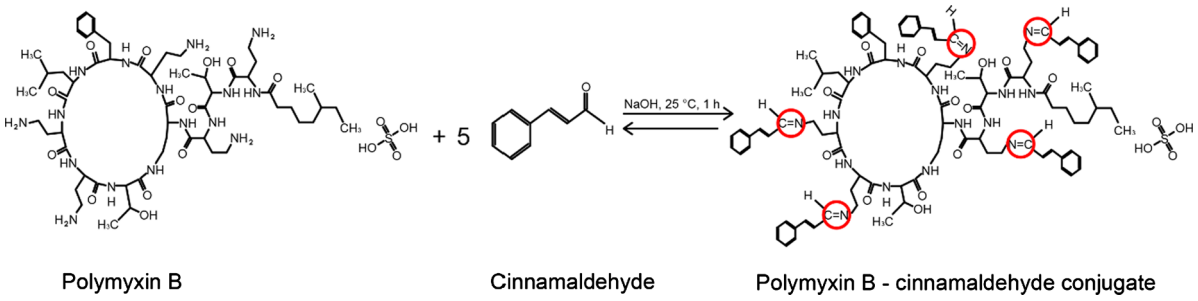

Fig. 1. Reaction between the primary amino groups of polymyxin B and carbonyl group of cinnamaldehyde under basic conditions.

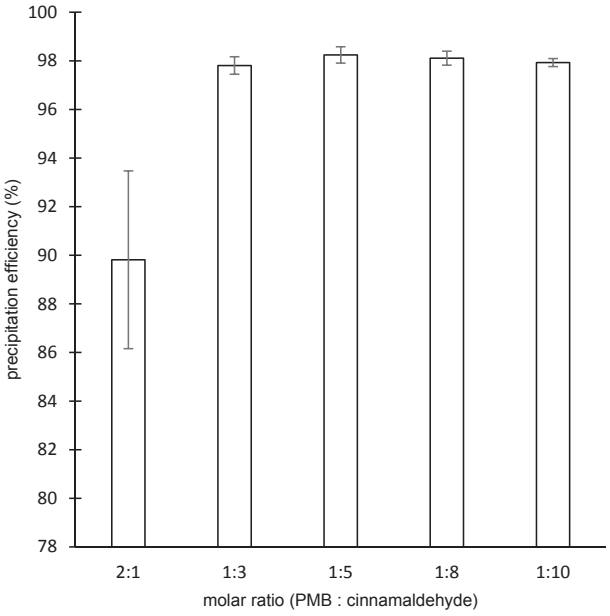

Fig. 2. Precipitation of polymyxin B (1 mg/ml) by reaction with cinnamaldehyde in molar ratio 2:1, 1:3, 1:5, 1:8 and 1:10. The precipitated PMB-cinnamaldehyde conjugates were isolated by centrifugation and the remaining PMB was analysed by RP HPLC. Precipitation efficiency was calculated by previous given Eq. (1). Data are shown as mean ± SD (n = 3).

3.2. Partitioning coefficient log P

In order to reach their site of action many drugs need to possess sufficiently high lipophilicity allowing them to permeate cell membranes. The partitioning coefficient log P (octanol/water) characterizes drug lipophilicity being essential for its efficient incorporation in oral lipophilic delivery systems such as SEDDS [2,21]. Log P represents the ratio between concentration of an unionized compound in n-octanol and its concentration in water at equilibrium [22]. In this study, log P of unmodified PMB was calculated to be −0.84. On contrary, cinnamaldehyde has a calculated log P of 1.98 (Chemicalize.org). After conjugation of PMB with cinnamaldehyde, results showed an almost 70,000 – fold increase in lipophilicity of PMB with a log scale increase from −0.84 to 4 (Fig. 4). Analyses of conjugates showed that a plateau in log P values is reached at molar ratios 1: ≥3.

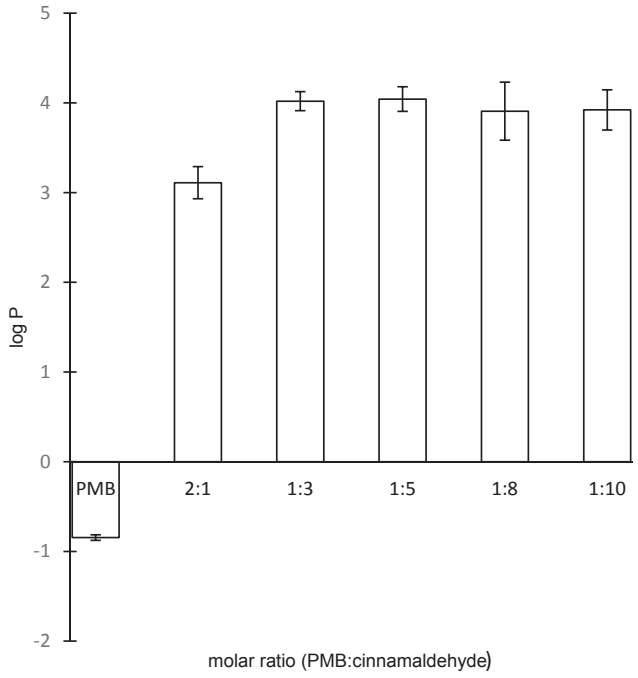

Fig. 4. Log P (partition coefficient) of PMB and conjugates in n-octanol/water mixture. Indicated values are mean ± SD.

3.3. Degree of substitution of PMB via TNBS assay

To calculate the degree of substitution, remaining free primary amino groups of PMB were quantified with 2,4,6,-trinitrobenzensulfonic acid (TNBS). The degree of substitution as well as the free amine content is listed in Table 1. Results showed a comparatively lower degree of substitution when conjugates were prepared in a molar ratio PMB to cinnamaldehyde of 2:1. In contrast, an up to 80% degree of substitution was achieved for conjugates formed in ratios 1: ≥3. These data are in good agreement with corresponding Log P values. As 80% of primary amino groups of PMB-cinnamaldehyde conjugate formed in 1:5 ratio were substituted (Table 1), this ratio was chosen for further experiments.

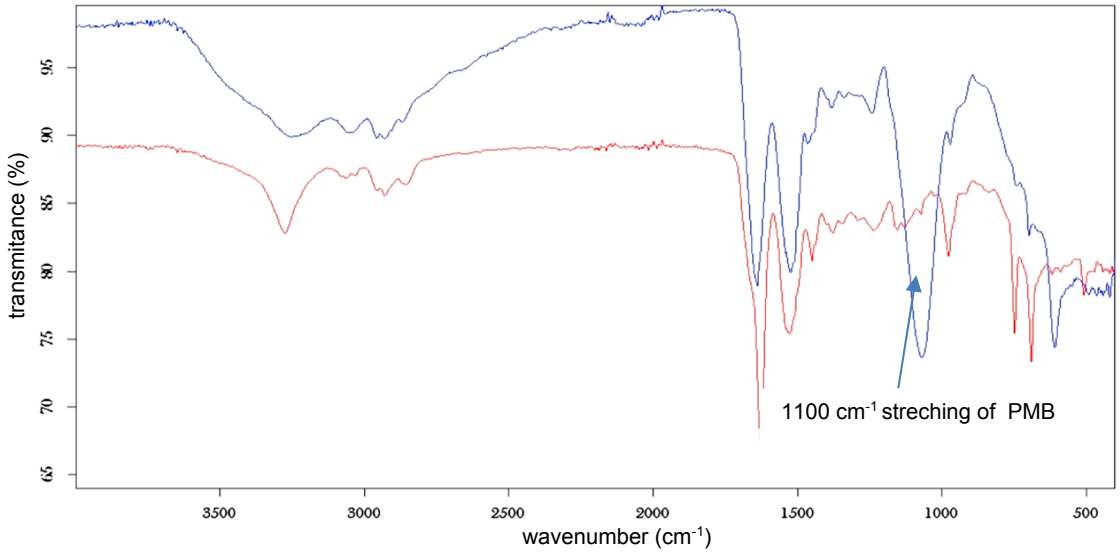

Fig. 3. IR spectrum of polymyxin B and polymyxin B-cinnamaldehyde conjugate.

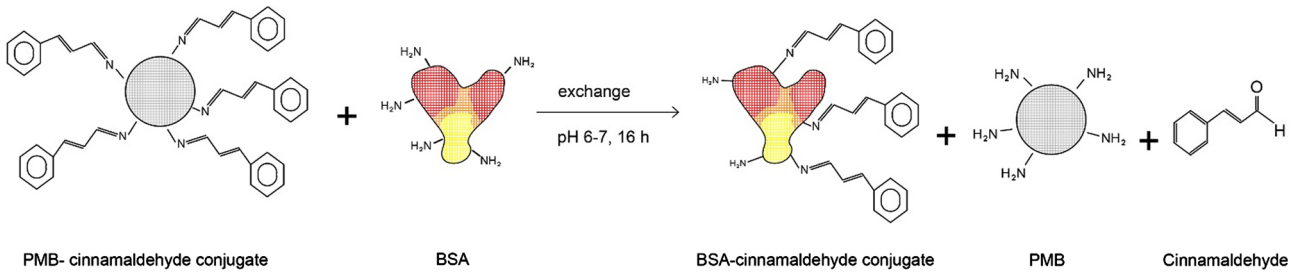

Fig. 5. Mechanism of exchange reaction between imine conjugates of PMB-cinnamaldehyde and amino groups of serum albumin.

3.4. Release of PMB from PMB-cinnamaldehyde conjugate

In order to investigate exchange reactions between the PMB-cinnamaldehyde conjugate and amino groups of serum albumin, bovine serum albumin (BSA) instead of human serum albumin was employed for financial reasons. To simulate the concentration of serum albumin in human blood being in the range of 0.53–0.75 mM, 4% (m/V) solution of BSA in water was utilized [16]. The mechanism is illustrated in Fig. 5. Results as shown in Fig. 6 indicate a release of more than 50% of PMB at pH 6, 6.5 and 7 within 16 h of incubation. During the incubation at pH 6, more than 70% of free PMB were released. The ANOVA test showed significant differences ($p < 0.05$) in the release of free PMB at pH 6 and pH 7. The dynamic of BSA interacting with the PMB-cinnamaldehyde conjugate is illustrated in Fig. 7a. In Fig. 7b–d results show a significant difference in the amount of released free PMB at pH 6, pH 6.5 and pH 7. On the one hand, BSA could push polymyxin B out of the conjugate (Fig. 7b–d) and on the other hand the conjugate could simply fall apart [15] and the cinnamaldehyde could be bound thereafter to serum albumin forming new conjugates. In either way the free amino groups of PMB could interact in the following with lipopolysaccharides of the outer membrane of Gram-negative bacteria in order to cause bacterial cell lysis and death. The significant differences in results between release studies performed at pH 6 and pH 7 would confirm the latter theory as conjugates fall more easily apart at acidic pH.

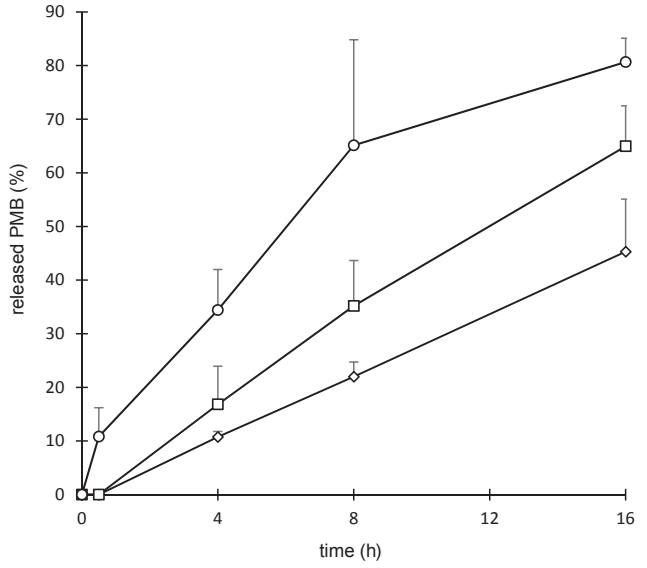

Fig. 6. The percentage of released polymyxin B was monitored at pH 6.0 (○), pH 6.5 (□) and pH 7.0 (◇), 37 °C for 16 h. The values are reported as mean ± SD (n = 3).

3.5. Development and characterization of SEDDS and log $D_{SEDDS/RM}$

Preliminary screening of PMB-cinnamaldehyde solubility in different organic solvents showed higher PMB-cinnamaldehyde solubility in protic solvents such as Transcutol, propylene glycol, Tween 20 and Cremophor EL compared to aprotic solvents of relatively low dielectric constant such as triglycerides. PMB-cinnamaldehyde conjugate was more soluble in Peceol containing mono-, di- and triglycerides of oleic acid where the monoesters are dominant and Capmul MCM containing mono- and diglycerides of caprylic/capric acid compared to Captex 300 containing triglycerides of caprylic/capric acid. PMB-cinnamaldehyde, however, showed low solubility in Capryol 90 containing propylene glycol monocaprylate and Captex 200 containing propylene glycol dicaprylate [23,24]. Based on solubility studies (Table 2) four different formulations were developed and examined regarding mean droplet

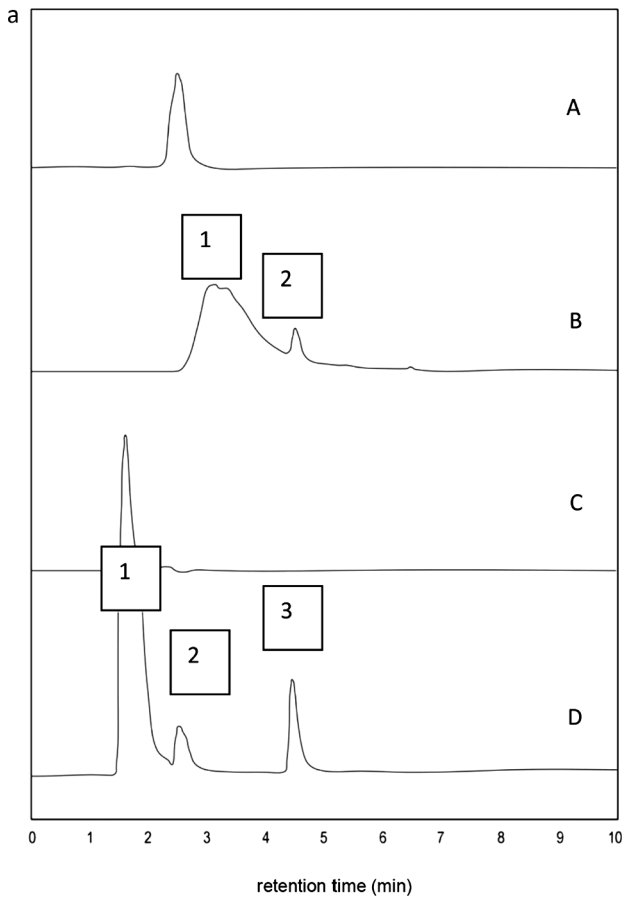

Fig. 7a. Dynamic of the standard HPLC chromatograms: (A) polymyxin B, detection wavelength 212 nm, (B) 1-PMB-cinnamaldehyde conjugate and 2-cinnamaldehyde, detection wavelength 328 nm, (C) bovine serum albumin, detection wavelength 212 nm and (D) 1-bovine serum albumin, 2-released free polymyxin B and 3-cinnamaldehyde, detection wavelength 212 nm.

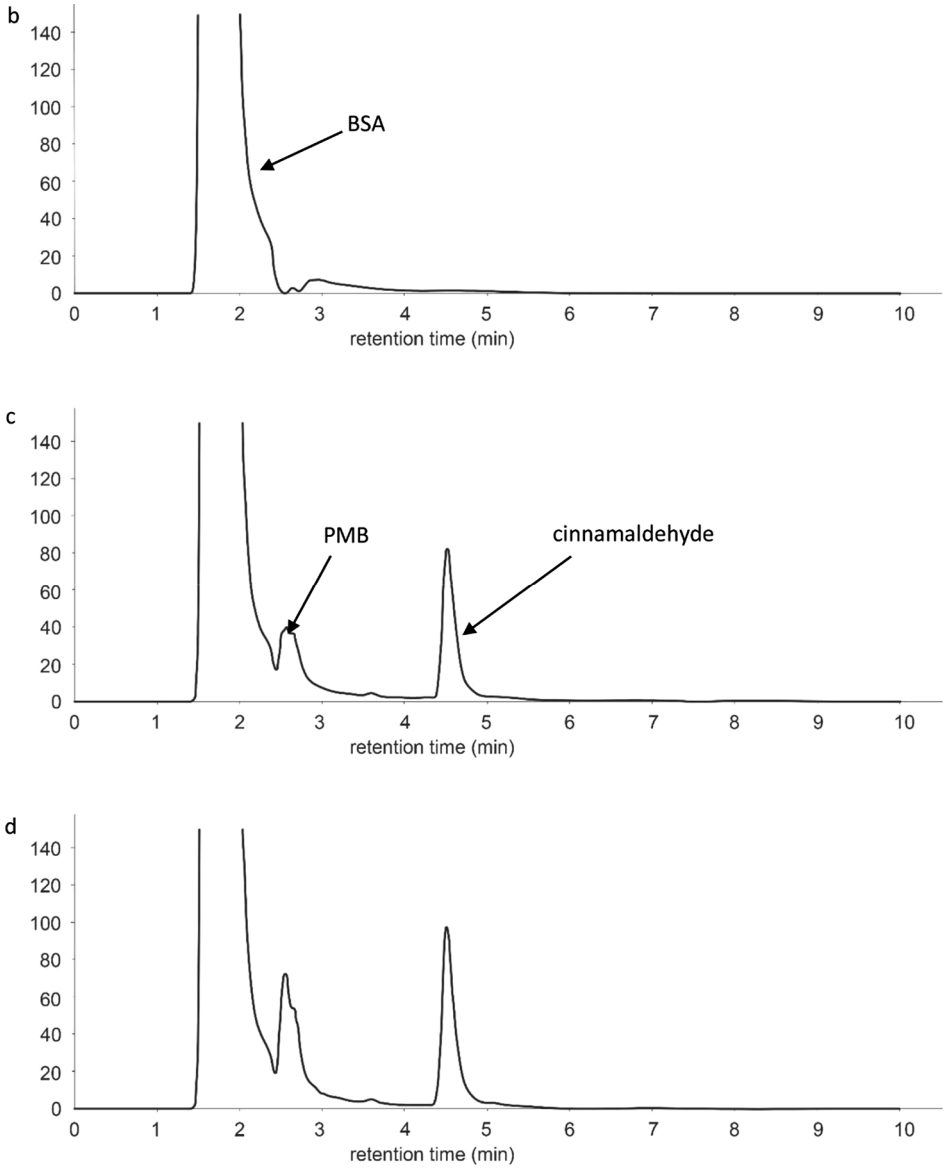

Fig. 7b–d. HPLC chromatograms of released PMB and cinnamaldehyde after incubation with BSA at time point 0 min (7b), 8 h (7c) and 16 h (7d) at pH 6, 37 °C and detection wavelength 212 nm.

size as well as polydispersity index (Table 3). Stability of the SEDDS pre-concentrate was confirmed through centrifugation, showing no phase separation of SEDDS formulations. The results demonstrated a mean droplet size of above 100 nm. Formulation C (Table 3) showed the highest mean droplet size and high PDI value. This observation might be attributed to the highly lipophilic composition of SEDDS C with a Peceol content of even 34.6%. The concentration of non-ionic surfactants in the four formulations was 27.2–45%. The required concentration of non-ionic surfactants in formulations is usually higher than 25% (Table 3) [25]. Obtained PDI values were in the range from 0.317 for formulation A to 0.388 for formulation D. SEDDS formulation D was chosen for further studies because of its constant size before and after conjugate loading and a PDI < 0.4. According to literature data PDI values of 0.1–0.25 indicate a narrow size distribution while a PDI greater than 0.5 refers to a broad distribution [26].

All formulations contained a relatively high amount of hydrophilic components like Capmul MCM, Cremophor EL and Tween 20. Payload of PMB-cinnamaldehyde conjugate formed in 1–5 M ratio of PMB to cinnamaldehyde was 3.6% in formulation D. Mean droplet size of loaded SEDDS D formulation was measured at time points 0 h and 4 h after emulsification and showed no difference to the corresponding unloaded SEDDS D formulation. The determined Log D of formulation D was 3.4.

3.6. Cell viability

The results of tested samples indicated no toxic effect of SEDDS, PMB alone and PMB-cinnamaldehyde in examined concentrations (Fig. 8). The values of cell viability were 101.31% for 0.1% SEDDS emulsion loaded with PMB-cinnamaldehyde, 103.02% for non-modified Polymyxin B and 103.24% for Polymyxin B-cinnamaldehyde conjugate. Indeed the values of all tested samples were higher than 100%. This observation might be explained by the impact of different solvents used for negative control (MEM without phenol red) and for samples (HBSS buffer).

4. Discussion

PMB-cinnamaldehyde conjugate was formed by covalent bonding between the electrophilic carbonyl group of cinnamaldehyde and the

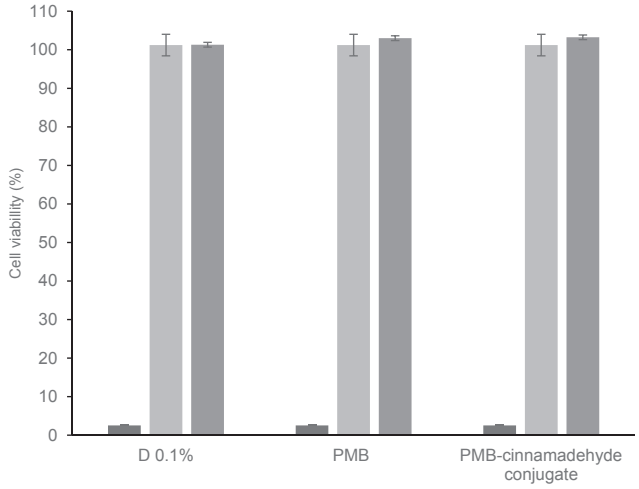

Fig. 8. Cell viability studies using Caco-2 cell lines; Formulation D, Polymyxin B and PMB-cinnamaldehyde conjugate were examined as follows: positive (■), negative (■) and sample cell viability (■). Indicated values are represented as means (n ≥ 3) ± SD.

nucleophilic NH_2 group of PMB. As proposed by Hugo Schiff, after nitrogen deprotonation, electrons from N—H bond are pushing the oxygen away from the carbon. Substitution of the oxygen atom in the carbonyl group with nitrogen results in the formation of an imine bond, also called Schiffs base [27]. As the electronegativity of the amino groups is 3.04 and electronegativity of the aldehyde group of cinnamaldehyde is 2.55, calculated ΔEn of 0.49 indicates a polar covalent bond having no equal sharing of electrons in the molecule. In order to show its mechanism of action, PMB should be released from the PMB-cinnamaldehyde conjugate in blood circulation. Thus, the reversibility of imine bond in this study has been confirmed by utilizing bovine serum albumin (BSA) and incubating it with PMB-cinnamaldehyde conjugate under physiological conditions in vitro. As a final product of this reaction BSA-cinnamaldehyde conjugates are formed and free PMB is released. The release of PMB after incubation with BSA (Fig. 6) could be triggered by exchange reactions with serum albumin or by hydrolysis. Generally, the concept of imine formation can be used for the development of different pH-triggered systems. Gu et al., for instance, have reported about a concept of formation of amphiphilic "stealth" polycations based on poly(ethylene glycol) (PEG)- poly-L lysine (PLL)-cholic acid (CA). They first attached cholic acid to poly-L-lysine. To obtain a methoxy poly(ethylene glycol) benzaldehyde conjugate (PEG-CHO) p-formylbenzoic acid was covalently attached to the free hydroxyl group of mPEG via esterification. In the following, an acid cleavable imine bond was formed between the aldehyde group of PEG-CHO and amino groups of PLL-CA. This acid cleavable imine linker was shown to detach PEG-CHO at endosomal low pH [28]. According to Ogris et al. conjugation of polycations to PEG provided evidence for a prolonged systemic circulation of PEGylated polycationic substructures [29]. Similar concept was also tested by Walker et al. They examined pH stability of polycation poly-L-lysine (PLL) bound via pH sensitive acylhydrazides or pyridylhydrazines to PEG at physiological and endosomal pH at 37 °C, confirming the reversibility of the hydrazine bond [30]. The increase of Log P of PMB-cinnamaldehyde conjugate permitted its incorporation into SEDDS delivery systems. Due to higher hydrophilic-lipophilic balance (HLB), monoesters such as Peceol and Capmul MCM demonstrated higher solubilizing properties compared to triglycerides. Furthermore, PEGylated non-ionic surfactants exhibiting HLB values between 12 and 18 such as Cremophor EL or Tween 20 demonstrated similar solubilizing properties for PMB-cinnamaldehyde conjugate. In addition, these surfactants are responsible for providing stable emulsions and proper size diameters < 500 nm [23,6]. In comparison to ionic surfactants, non-ionic surfactants are more commonly used for SEDDS development due to less toxicity, lower critical micelle concentration and lower concentration required to reduce interfacial energy representing one of the main factors required for SEDDS stability [25]. SEDDS were loaded with PMB-cinnamaldehyde in concentration of 3.6%, showing log D value of 3.4. Caco 2- cell lines, isolated from a human colon carcinoma, are commonly used to predict intestinal permeability as well as to screen cytotoxicity of drug delivery systems. Since the average small intestinal passage time is 4 h, incubation of SEDDS was conducted for this time period. In comparison to positive control (Triton X), PMB alone or conjugated to cinnamaldehyde did not show toxic effects in terms of cell viability in applied concentrations [21,4,31,32]. In an in vivo study in rats, oral toxicity of PMB was evaluated. Rats were treated with a single dose of PMB in the range of 62.5–300 mg kg^{-1}. Those rats that were dosed with 62.5 mg kg^{-1} survived the treatment with PMB, confirming that the LD for rats is lower than that reported for mice (790 mg/kg) [33]. Shreaz et al. have reported LD_{50} of cinnamaldehyde between 0.6 and 2 g/kg for the oral and dermal route. Moreover, they have stated that the LD_{50} value when taken orally in for instance white rats, white mongrel mice and guinea pigs was 3.4 g/kg. In contrast, the value was 2.318 g/kg when mice were treated with cinnamaldehyde by intraperitoneal route [34]. According to above mentioned studies, low toxicity of both PMB and cinnamaldehyde was confirmed.

5. Conclusion

In the present work, we used imine bond formation as a tool to increase lipophilicity of the hydrophilic antibiotic peptide PMB. Imine bond was formed between the primary amino groups of PMB and carbonyl group of cinnamaldehyde. To simulate in vivo liberation of PMB and binding of cinnamaldehyde to serum albumin after having reached the systemic circulation, PMB-cinnamaldehyde conjugate was incubated with serum albumin at pH 6–7 and 37 °C for 16 h. Conjugate formed via imine bond seems to be more stable compared to hydrophilic ion pairs, but still unstable enough to fall apart in the systemic circulation. In addition, this pH 6–7 cleavable bond promises great potential for incorporation of even bigger hydrophilic macromolecules by using the proposed concept. Imine bond formation showed already great potential for various biological applications and has been investigated in numerous studies mostly because of its reversible nature. Regarding future research, efforts could be focused on membrane permeability studies of such imine conjugates incorporated in SEDDS.

Acknowledgements

The authors would like to thank to Gattefossé and Abitec companies for their technical support.

Declaration of Competing Interest

There is no conflict of interest associated with this research.

References

[1] R.N. Gursoy, S. Benita, Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs, Biomed. Pharmacother. 58 (3) (2004) 173–182.
[2] C. Menzel, et al., In vivo evaluation of an oral self-emulsifying drug delivery system (SEDDS) for exenatide, J. Control. Release 277 (2018) 165–172.
[3] S. Bonengel, et al., Impact of different hydrophobic ion pairs of octreotide on its oral bioavailability in pigs, J. Control. Release 273 (2018) 21–29.
[4] O. Zupančič, 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.
[5] S. Hauptstein, F. Prüfert, A. Bernkop-Schnürch, Self-nanoemulsifying drug delivery systems as novel approach for pDNA drug delivery, Int. J. Pharm. 487 (1) (2015) 25–31.
[6] A. Mahmood, A. Bernkop-Schnurch, SEDDS: A game changing approach for the oral administration of hydrophilic macromolecular drugs, Adv. Drug Deliv. Rev. (2018).

[7] A. Bernkop-Schnürch, A. Jalil, Do drug release studies from SEDDS make any sense? J. Control. Release 271 (2018) 55–59.
[8] K. Sirinan, M.N. Chaur, L. Jean-Marie, Kinetic selectivity and thermodynamic features of competitive imine formation in dynamic covalent chemistry, Chem. – A Eur. J. 23 (46) (2017) 11108–11118.
[9] C. Godoy-Alcántar, A.K. Yatsimirsky, J.M. Lehn, Structure-stability correlations for imine formation in aqueous solution, J. Phys. Organic Chem. 18 (10) (2005) 979–985.
[10] V.K. Rao, et al., Synthesis of Schiff's bases in aqueous medium: a green alternative approach with effective mass yield and high reaction rates, Green Chem. Lett. Rev. 3 (3) (2010) 217–223.
[11] A.L. Kwa, V.H. Tam, M.E. Falagas, Polymyxins: a review of the current status including recent developments, Ann. Acad. Med. Singapore 37 (10) (2008) 870–883.
[12] E.K. Schneider-Futschik, et al., Sputum active polymyxin lipopeptides: activity against cystic fibrosis pseudomonas aeruginosa isolates and their interactions with sputum biomolecules, ACS Infect. Dis. 4 (5) (2018) 646–655.
[13] A.M. George, P.M. Jones, P.G. Middleton, Cystic fibrosis infections: treatment strategies and prospects, FEMS Microbiol. Lett. 300 (2) (2009) 153–164.
[14] F. Schaufelberger, B.J. Timmer, O. Ramström, Principles of dynamic covalent chemistry, in: W. Zhang, Y. Jin (Eds.), Dynamic Covalent Chemistry, 2017. doi: https://doi.org/10.1002/9781119075738.ch1.
[15] K.C. Waterman, et al., Hydrolysis in pharmaceutical formulations, Pharm. Dev. Technol. 7 (2) (2002) 113–146.
[16] A. Bujard, et al., Modification of a PAMPA model to predict passive gastrointestinal absorption and plasma protein binding, Eur. J. Pharm. Sci. 77 (2015) 273–278.
[17] B. Jansson, et al., Quantitative analysis of colistin A and colistin B in plasma and culture medium using a simple precipitation step followed by LC/MS/MS, J. Pharm. Biomed. Anal. 49 (3) (2009) 760–767.
[18] J. Griesser, et al., Hydrophobic ion pairing: key to highly payloaded self-emulsifying peptide drug delivery systems, Int. J. Pharm. 520 (1) (2017) 267–274.
[19] P. Cayot, G. Tainturier, The quantification of protein amino groups by the trinitrobenzenesulfonic acid method: a reexamination, Anal. Biochem. 249 (2) (1997) 184–200.
[20] N.A. Efiana, et al., Improved mucoadhesive properties of self-nanoemulsifying drug delivery systems (SNEDDS) by introducing acyl chitosan, Int. J. Pharm. 519 (1–2) (2017) 206–212.
[21] O. Zupančič, et al., Development and in vitro evaluation of an oral SEDDS for desmopressin, Drug Deliv. 23 (6) (2016) 2074–2083.
[22] K. Valkó, Application of high-performance liquid chromatography based measurements of lipophilicity to model biological distribution, J. Chromatogr. A 1037 (1) (2004) 299–310.
[23] J. Griesser, et al., Hydrophobic ion pairing: Key to highly payloaded self-emulsifying peptide drug delivery systems, Int. J. Pharm. 520 (1–2) (2017) 267–274.
[24] I. Nazir, et al., Self-emulsifying drug delivery systems: Impact of stability of hydrophobic ion pairs on drug release, Int. J. Pharm. 561 (2019) 197–205.
[25] C.W. Pouton, Formulation of self-emulsifying drug delivery systems, Adv. Drug Deliv. Rev. 25 (1) (1997) 47–58.
[26] L. Wu, J. Zhang, W. Watanabe, Physical and chemical stability of drug nanoparticles, Adv. Drug Deliv. Rev. 63 (6) (2011) 456–469.
[27] H. Schiff, Eine neue Reihe organischer Diamine, Justus Liebigs Annalen der Chemie 140 (1) (1866) 92–137.
[28] J. Gu, et al., pH-Triggered Reversible "Stealth" Polycationic Micelles, Biomacromolecules 9 (1) (2008) 255–262.
[29] M. Ogris, et al., PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery, GeneTher 6 (4) (1999) 595–605.
[30] G.F. Walker, et al., Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo, Mol. Ther. 11 (3) (2005) 418–425.
[31] P. Bu, et al., Assessment of cell viability and permeation enhancement in presence of lipid-based self-emulsifying drug delivery systems using Caco-2 cell model: Polysorbate 80 as the surfactant, Eur. J. Pharm. Sci. 99 (2017) 350–360.
[32] G. Meca, et al., Study of the potential toxicity of enniatins A, A(1), B, B(1) by evaluation of duodenal and colonic bioavailability applying an in vitro method by Caco-2 cells, Toxicon 59 (1) (2012) 1–11.
[33] G. Coppi, et al., Toxicity and gut associated lymphoid tissue translocation of polymyxin B orally administered by alginate/chitosan microparticles in rats, J. Pharm. Pharmacol. 60 (1) (2008) 21–26.
[34] S. Shreaz, et al., Cinnamaldehyde and its derivatives, a novel class of antifungal agents, Fitoterapia 112 (2016) 116–131.