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Improved Intestinal Mucus Permeation of Vancomycin via Incorporation Into Nanocarrier Containing Papain-Palmitate

Nuri Ari Efiana [1, 2], Aida Dizdarević [1], Christian W. Huck [3], Andreas Bernkop-Schnürch [1, *]

[1] Department of Pharmaceutical Technology, Institute of Pharmacy, Leopold-Franzens University Innsbruck, Innrain 80/82, A-6020 Innsbruck, Austria
[2] Department of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Ahmad Dahlan, Jl. Prof. Dr. Soepomo, S.H., Janturan, Warungboto, Umbulharjo, Yogyakarta 55164, Indonesia
[3] Institute for Analytical Chemistry and Radiochemistry, University Innsbruck, Innrain 80/82, A-6020 Innsbruck, Austria

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ABSTRACT

The aim of this study was to improve intestinal mucus permeation of a peptide antibiotic via incorporation into papain-palmitate–modified self-emulsifying drug delivery systems (SEDDS) as nanocarrier. Vancomycin as a peptide antibiotic was lipidized by hydrophobic ion pair formation using sodium bis-2-ethylhexyl-sulphosuccinate before incorporation in SEDDS comprising Capmul MCM, propylenglycol, and Kolliphor EL (2:1:2). As mucolytic agent, 0.5% papain-palmitate was introduced in SEDDS formulation containing the vancomycin-sodium bis-2-ethylhexyl-sulphosuccinate ion pair. The formulation was evaluated regarding droplet size, zeta potential, and cytotoxicity using Caco-2 cells previous to intestinal mucus permeation studies using Transwell diffusion and rotating tube method. The hydrophobic ion pair product yielded from surfactant to drug ratio of 3:1 provided a 25-fold increase in lipophilicity, drug payload in SEDDS of 5%, and log $D_{SEDDS/release\ medium}$ of 2.2. The formulation exhibited a droplet size and zeta potential of 221.5 ± 14.8 nm and −4.2 ± 0.8 mV, respectively. Cytotoxicity study showed that SEDDS formulations were not toxic. Introducing 0.5% papain-palmitate increased the mucus permeability of SEDDS 2.8-fold and 3.3-fold in Transwell diffusion and rotating tube studies, respectively. According to these results, papain decorated SEDDS might be a potential strategy to improve the mucus permeating properties of peptide antibiotics.

Introduction

During the last decade, the worldwide consumption of antibiotics has been raising for all classes, particularly for broad-spectrum penicillins,[1] parallelly with a growing number of infectious diseases.[2] The abuse of antimicrobial agents causes bacteria to evolve such as by gene mutations decreasing their sensitivity to antibiotics.[3] This phenomenon is referred to as antimicrobial resistance (AMR) which increases medical costs and mortality.[4] The growing number of AMR triggers various attempts to discover alternative antibiotics. Among these alternatives, peptide antibiotics have shown potential in particular for treatment of persistent bacterial infections,[5] as they struggle against bacteria via multiple mechanisms resulting in a lower incidence of resistance.[6]

Peptide-based antibiotics are a class of antimicrobial agents that contain 2 or more connected amino acids. They can be produced in multicellular organisms or synthesized by bacteria, fungi, and streptomycetes. The latter are widely used in infection treatment such as polymyxin including colistin, gramicidin, and bacitracin[7] being produced in large quantities by *Paenibacillus polymyxia*,[8] *Bacillus brevis*,[9] and *Bacillus licheniformis*,[10] respectively. Peptide-based antibiotics containing sugar moieties are referred to as glycopeptides including vancomycin and teicoplanin[7] and are produced by *Amycolatopsis orientalis*[11] and *Actinoplanes teichomyceticus*,[12] respectively.

On account of the high molecular mass of even small molecule peptide-based antibiotics with more than 1000 Da and low lipophilicity owing to the nature of amino acids, these peptide antibiotics have to be administered parenterally for treatment of systemic infections. Moreover, the low oral bioavailability of peptide antibiotic is also caused by several barriers of gastrointestinal tract such as an acidic gastric environment causing instability and deactivation of peptide,[13,14] gastrointestinal protease cleaving

Conflicts of interest: None.

* *Correspondence to:* Andreas Bernkop-Schnürch (Telephone: +43 512 507 58601).
E-mail address: andreas.bernkop@uibk.ac.at (A. Bernkop-Schnürch).

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peptide bonds,[14,15] the mucus gel layer hindering peptide to reach the underlaying epithelium, and the intestinal membrane confining peptide to enter the systematic circulation.[16] To overcome these barriers, new concepts are needed, in particular by application of nanocarrier protecting the peptide from gastrointestinal environment.

Although nanocarrier oral delivery could solve the first and second problems mentioned above, the routine regenerated mucus is still existed triggering further research to formulate mucus penetrating nanocarriers by surface modification. Previous research showed that surface modification of silica nanoparticles affected mucus permeability. Cationic-charged nanoparticles were bonded more tightly to mucus compared with anionic ones resulting in lower permeation.[17] Another research also demonstrated that surface modification of thiolated silica nanoparticles using various side chains of polyoxazoline could increase mucus permeability. The higher hydrophilicity and hydration of surface-modified thiolated silica nanoparticles resulted in such greater permeability.[18] Increasing mucus permeability also could be reached by introducing proteolytic enzyme into nanoparticles.[19]

Vancomycin, a peptide-based antibiotic studied in present research, mostly acts against gram-positive bacteria by a high affinity binding of the heptapeptide moiety, in particular N-methyl leucine residue, to D-Ala-D-Ala C-terminus of the bacterial pentapeptide, the part of UDP-N-acetylmuramyl-pentapeptide, a substrate in peptidoglycan synthesis.[20] Blocking this enzyme results in the inhibition of cell membrane biosynthesis.[21] Currently, the oral use of vancomycin is only intended for local infections of the gastrointestinal tract such as for treatment of *Clostridium difficile* infections in the colon.[22] As an intravenous injection, vancomycin is one of the most important antibiotics in combating systemic infections by Enterococci and Staphylococci including methicillin-resistant *Staphylococcus aureus*.[23] The parenteral administration, however, is inconvenient for patients and occasionally even risky. In case of certain infections, long-term treatments with orally administered vancomycin might even be more efficient than intravenous injection. Oral delivery systems for vancomycin would therefore be highly beneficial. Even though vancomycin exhibits stability against gastrointestinal proteases owing to its complex structure bearing also nonpeptide groups protecting the amino acid backbone, the hydrophilic and polycationic nature of vancomycin causes low mucus permeation due to hydrogen bonding and ionic interactions with sialic acid and sulfonic acid substructures of mucus.[24] Furthermore, vancomycin is poorly absorbed resulting in an oral bioavailability of less than 5%.[25-27] With the intention of overcoming these barriers, new concepts are needed.

Recently, self-emulsifying drug delivery systems (SEDDS) were adapted as nanocarriers for the oral administration of hydrophilic macromolecular drugs. Via hydrophobic ion pairing, the lipophilic character of these drugs can be strongly increased so that they can be incorporated in the oily phase of SEDDS. Once SEDDS containing the drug disperse in the intestinal fluid, the formed droplets face the intestinal mucus. This mucus gel layer obstructs nanocarriers to reach underlying intestinal cells. Previous research showed that introducing trypsin as mucolytic agent in SEDDS formulations increased mucus permeation effectively.[28] So far, the concept of mucolytic agent modified SEDDS as nanocarriers for oral peptide antibiotics delivery, however, has not been utilized.

It was therefore the aim of this study to improve intestinal mucus permeation of peptide antibiotics, for instance vancomycin, via incorporation into papain-palmitate—modified SEDDS as nanocarrier. Because SEDDS comprise lipophilic solvents, surfactants and cosolvent exhibiting lipophilic properties, vancomycin was lipidized by formation of hydrophobic ion pairs (HIP) with anionic surfactants prior to be incorporated. Papain-palmitate as mucolytic agent was introduced into SEDDS containing the most appropriate vancomycin-surfactant ion pair. The resulting nanoemulsion droplets were evaluated regarding droplet size, drug stability against papain-palmitate featuring on SEDDS droplets and toxicity on Caco-2 cells. Furthermore, the mucus permeability was determined by Transwell diffusion and rotating tube method.

Experimental

Materials

Vancomycin hydrochloride was obtained from Sigma Aldrich (Hamburg, Germany). Sodium dodecyl benzene sulfonate (SDBS) and sodium bis-2-ethylhexyl-sulfosuccinate (SBS) from Sigma Aldrich (Germany) were used as surfactants for the preparation of HIP. Oleic acid and oleum lini virginium from Gatt-Koller GmbH (Absam, Austria), Capmul MCM and Captex 355 from ABITEC Corporation (Columbus, OH), Capryol 90 and Labrafil M 1944 CS from Gattefosse (Lyon, France), Miglyol 840 from IOI Oleo GmbH (Hamburg, Germany), and SP Crodamol GTCC MBAL-LQ-(MV) from Croda (Barcelona, Spain) were used as solvents for SEDDS formulations. Propylenglycol and Kolliphor EL from Sigma Aldrich (Vienna, Austria) were used as co-solvent and surfactant, respectively. Fluorescein diacetate (FDA) was obtained from Sigma Aldrich (Vienna, Austria). Papain (from Carica papaya) from Sigma Aldrich (Vienna, Austria) was used as mucolytic agent. Palmitoyl chloride from Acros Organics (Morris, NJ) was used as acylating agent. Mucus was collected from porcine intestine having been obtained freshly from a local slaughter house. Mucus was cleaned from remaining feces and food.

Synthesis of Papain-Palmitate Conjugate

Synthesis of papain-palmitate was performed by modifying a method having been described previously by Xue et al.[29] Briefly, one-part volume of palmitoyl chloride solution in acetone with a concentration of 0.4 M was added dropwise to 40-part volume of papain solution in 0.1 M phosphate buffer pH 8.0 with a concentration of 3 mg/mL. The reaction was performed at room temperature under mild stirring for 90 min, and the pH was adjusted to 8.0, followed by dialysis against water for 24 h and lyophilization.

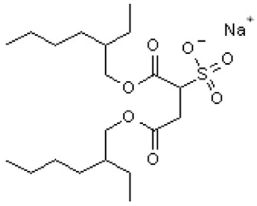
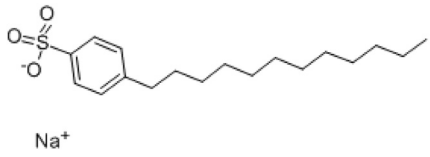
Quantification of Vancomycin

Concentration of vancomycin was determined via HPLC (HITACHI Elite LaChrom; Hitachi, Tokyo, Japan) using YMC column C4 (250 × 4.6 mm) as a stationary phase. The column temperature was set up at 25°C, and UV detector was used at a wavelength of 214 nm. Isocratic elution was used for quantification of vancomycin in precipitation efficiency (PE) studies. The elution was set up at a rate of 1 mL/min using a mixture of acetonitrile and 0.05% trifluoroacetic acid (TFA) in water at a volume ratio of 75:25. The gradient method was used for partition coefficient determination and stability studies. Eluent A: 0.05% TFA in water; eluent B: a mixture of 0.05% TFA in water and acetonitrile (1:19); gradient from 100%A/0% B at 0 min decreased to 85%A/15%B at 8 min and return to 100%A/0% B up to 15 min.

Formation of Hydrophobic Ion Pair

Formation of HIP was conducted according to a method described previously by Griesser et al.[30] Vancomycin hydrochloride was dissolved in a final concentration of 0.7 mM in 0.01 M HCl. Subsequently, SBS as well as SDBS solution in water at a concentration of 0.1 M were added dropwise in ratios as listed in Table 1.

Table 1
Molar Ratio Between Vancomycin and Surfactant Including Sodium Bis-2-Ethylhexyl-Sulphosuccinate (SBS) and Sodium Dodecylbenzene Sulfonate (SDBS)

Peptide	Tested Surfactants	Molar Ratio (Vancomycin:Surfactant)
Vancomycin hydrochloride, net positive charges: 2	SBS, net negative charge: 1 	2:1 1:1 1:2 1:3 1:5 1:7
	SDBS, net negative charge: 1 	2:1 1:1 1:2 1:3 1:5 1:7

The reaction was conducted for 2 h using thermomixer at the rate of 500 rpm. Afterward, HIP was separated via centrifugation (High-Speed MiniSpin™ Microcentrifuge Fisher Scientific; Eppendorf North America, Hauppauge, NY) at 10,530 rcf for 10 min. The sediment containing HIP was rinsed with 0.01 M HCl before lyophilization. The products were stored at 4°C. To calculate PE, the concentration of uncomplexed vancomycin in supernatant was determined using HPLC as described above. PE was determined as a percentage of vancomycin complexed to total vancomycin introduced in HIPs formation and calculated according to Equation 1 as follows. In such equation, Vi and Vr represent initial total amount of vancomycin hydrochloride and free vancomycin hydrochloride remaining in the supernatant after HIP formation, respectively.

$$PE\ (\%) = \frac{Vi - Vr}{Vi} \times 100 \quad (1)$$

Evaluation of Zeta Potential During HIP Formation

Determination of zeta potential during HIP formation was performed as follows.[30] Surfactant solution was added dropwise to 0.5 mL of 1 mg/mL vancomycin hydrochloride solution in 0.01 M HCl in various molar ratios as listed in Table 1. Afterward, the mixture was kept in thermomixer at the rate of 500 rpm for 2 h prior to zeta potential measurement (Zetasizer Nano-ZSP; Malvern Instruments, Worcestershire, UK).

Selection of Various Solvents for SEDDS Formulation

Various organic solvents such as Capmul MCM, Captex 355, oleum lini virginium, Miglyol 840, Capryol 90, oleic acid, Labrafil 1944, and Crodamol were used in solvent selection by evaluating the capability to dissolve HIP. The experiment was carried out by dissolving a certain amount of HIP in 1 mL of oil using vortex for 5 min followed by sonication (Bandelin Sonorex at a frequency of 35 kHz) for 15 min. Subsequently, the samples were kept in thermomixer at the rate of 500 rpm at room temperature for 24 h. Afterward, the samples were centrifuged at 10,530 rcf for 5 min prior to sediment observation.

Preparation and Characterization of SEDDS Formulations

Three compositions of SEDDS as listed in Table 2 were prepared by mixing the components using vortex for 10 min followed by incubation in thermomixer at room temperature for 1 h. Subsequently, SEDDS were dispersed in 20 mM phosphate buffered saline (PBS) pH 6.8. The droplet size and polydispersity index (PI) were determined using Zetasizer.

SEDDS containing vancomycin-SBS ion pair (Van-SBS-SEDDS) was prepared first by dissolving HIP in Capmul MCM at a concentration of 12.5% using thermomixer at the rate of 1000 rpm at 20°C overnight. Afterward, co-solvent and surfactant were added gradually to the HIP solution. The mixture was blended using vortex for 15 min followed by sonication for 30 min and kept in thermomixer at the rate of 500 rpm for 2 h. All processes were conducted at room temperature.

SEDDS containing vancomycin-SBS ion pair and 0.5% of papain-palmitate were prepared as follows. Papain-palmitate and vancomycin-SBS ion pair (Van-SBS) were dissolved in Capmul MCM separately in a mass ratio of 1:20 and 1:6, respectively. Briefly, papain-palmitate was dispersed in Capmul MCM using vortex for 20 min, continued by sonication for 1 h and shaking in thermomixer at the rate of 1500 rpm overnight at 20°C. Afterward, solution of Van-SBS in Capmul MCM was added and mixed using vortex for 20 min followed by sonication for 60 min at room temperature. Finally, co-solvent and surfactant were added gradually to oil phase

Table 2
Composition of SEDDS Formulation F1, F2, and F3 Resulting in Droplet Size and Polydispersity Index (PI) When Being Diluted 1:100 in 20 mM PBS pH 6.8

Formulation	Composition of SEDDS (%)			Droplet Size (nm)	PI
	Oil (Capmul MCM)	Surfactant (Kolliphor EL)	Co-solvent (Propylenglycol)		
F1	30	40	30	202.6 ± 16.9	0.3 ± 0.0
F2	40	40	20	257.7 ± 9.2	0.3 ± 0.0
F3	40	30	30	775.6 ± 72.7	0.5 ± 0.1

Indicated Values are Means of 3 Experiments ±SD
SEEDS, self-emulsifying drug delivery system.

containing Van-SBS and papain-palmitate. The mixing procedure was the same as for the preparation of Van-SBS SEDDS. Droplet size, PI, and zeta potential of all formulations were determined immediately and 4 h after dispersing in 20 mM PBS pH 6.8 at a concentration of 1% using a zeta sizer.

Physical stability of SEDDS composition was observed firstly by centrifugation method. SEDDS dispersion in 20 mM PBS pH 6.8 at a concentration of 1% were centrifuged at 3500 rpm for 30 min. SEDDS composition was assigned to be stable if there is neither any separation nor sedimentation. Second, a heating-cooling stability study was performed with the same SEDDS dispersions as described above with 3 cycles of cooling in refrigerator (4°C) and heating (40°C) with exposure to each temperature for 48 h followed by physical evaluation of SEDDS precipitation and phase separation. The stress physical stability study with the same SEDDS dispersions as described above was also conducted by 3 cycles of freeze-thaw between −20°C and 25°C for 48 h, respectively.

Determination of Partition Coefficient

Determination of Log P

Log P of vancomycin and Van-SBS was determined by quantifying the concentration of vancomycin as well as Van-SBS in octanol and water phase after the partition process had reached equilibrium. Briefly, about 1 mg of vancomycin hydrochloride or of Van-SBS was dissolved in a mixture of 0.5 mL of octanol and 0.5 mL of water. Subsequently, the mixture was kept in thermomixer at 37°C at the rate of 300 rpm for 24 h, followed by centrifugation at the rate of 10,530 rcf for 10 min for phase separation. The concentration (C) of vancomycin hydrochloride as well as Van-SBS in both phases was determined using HPLC as described previously. The octanol phase was diluted using methanol prior to injection for HPLC analyses. The following Equation 2 was used for log P calculation.

$$Log\ P_{n-octanol/water} = log \frac{(C\ in\ octanol\ phase)}{C\ in\ water\ phase} \quad (2)$$

Determination of Log D

Log D between SEDDS preconcentrate and release medium provides an accurate value for the release behavior of drugs from SEDDS.[31] Excess amounts of Van-SBS in SEDDS preconcentrate and in 20 mM PBS pH 6.8 serving as release medium were separately agitated for 24 h followed by centrifugation at 10,530 rcf for 10 min. The solubility (Cs) of Van-SBS in SEDDS preconcentrate and PBS was defined as drug concentration in the supernatant determined by HPLC using the same method as used for log P determination, and log $D_{SEDDS/release\ medium}$ was calculated using Equation 3 as follows.

$$Log\ D_{SEDDS/release\ medium} = log \frac{(Cs\ in\ SEDDS)}{Cs\ in\ PBS} \quad (3)$$

Vancomycin Stability Against Papain in SEDDS Dispersion

First, 1 mL of Van-SBS-SEDDS and Van-SBS-SEDDS containing papain-palmitate dispersion in 20 mM PBS pH 6.8 at a concentration of 1%, as well as papain-palmitate modified SEDDS (Pap-SEDDS) dispersion containing vancomycin hydrochloride as control were incubated at 37°C in thermomixer and stirred at 500 rpm for 180 min. At scheduled time (0, 15, 30, 60, 120, and 180 min), 100 μL of the dispersion were withdrawn followed by the addition of 100 μL of TFA 2% to stop the enzymatic reaction. Afterward, the samples were centrifuged at 10,530 rcf for 5 min. The concentration of remaining Van-SBS as well as vancomycin was determined by HPLC as described above.

Cytotoxicity Study Using Caco-2 Cells

Resazurin assay was conducted to confirm the cytotoxicity of SEDDS formulations. Caco-2 cells were cultivated in a 24-well plate using red minimum essential medium (containing 10% (v/v) fetal calf serum and 100 units/0.1 mg/L penicillin solutions) in the incubator at 37°C. During 2 weeks of cultivation, the medium was renewed every 2 days to provide the elements for cell growth. The experiment was initiated by washing the Caco-2 cells using prewarmed Hank's balanced salt solution (HBSS) followed by incubation at 37°C for 30 min. Subsequently, HBSS was replaced with the 0.1% dispersion of samples in HBSS including SEDDS, Van-SBS-SEDDS, Van-SBS-SEDDS containing papain-palmitate, white-minimum essential medium as a negative control and Triton-X 100 as a positive control. All samples were incubated at 37°C for 4 h, continued by removing the samples and rinsing the cells using HBSS. Afterward, 250 μL of 20-fold diluted resazurin using PBS pH 6.8 were added and incubated at 37°C for 3 h. Cell viability was determined by measuring the fluorescence intensity of sample (As) and negative control (Ac) at the excitation wavelength of 540 nm and the emission wavelength of 590 nm using a spectrophotometer (Tecan infinite M200 spectrophotometer; Tecan Austria GmbH, Grödig, Austria). Cell viability was calculated using Equation 4 as follows:

$$Cell\ viability\ (\%) = \frac{As}{Ac} \times 100 \quad (4)$$

Permeation Study

For the purpose of permeation study, FDA was incorporated into SEDDS as control, Van-SBS-SEDDS as well as Van-SBS-SEDDS containing papain-palmitate at saturated concentration. Briefly, FDA was mixed with SEDDS formulation in a ratio of 1:50 by agitation using thermomixer at the rate of 1000 rpm at 20°C for 24 h. Afterward, the mixture was centrifuged at 10,530 rcf for 5 min. The supernatant containing saturated FDA was withdrawn and used for permeation studies.

Permeation Study via Transwell Diffusion

To evaluate mucus permeability of SEDDS, Transwell diffusion study was performed as described previously.[32] SEDDS containing FDA were dispersed in 20 mM PBS pH 6.8 at 1% concentration. Two hundred and fifty microliter of these dispersions were transferred in donor compartment of Transwell plates (24-well plates, Greiner-BioOne, Kremsmünster, Austria) containing 50 μL of mucus, whereas the acceptor compartment was filled with 500 μL of 20 mM PBS pH 6.8. The permeation was performed in an incubator at 37°C under agitation using shaking board (Vibramax 100; Heidolph Instruments, Schwabach, Bavaria, Germany) at 300 rpm. Aliquots of 100 μL were withdrawn from the acceptor compartment at 0, 1, 2, 3, and 4 h and substituted with the same volume of preheated 20 mM PBS pH 6.8. Afterward, 20 μL of 5 M NaOH was added to each withdrawn sample followed by incubation in a water bath at 37°C for 30 min for quantification of the liberated fluorescence using microplate reader Tecan at an excitation and emission wavelength of 485 nm and 535 nm, respectively. As 100% control, the permeation experiment of each FDA-labeled formulation was also performed without mucus.

Permeation Study via Rotating Tube Method

Rotating tube was performed as described previously.[33] The size of the tube was 4 mm in diameter and 40 mm in length. Briefly, the

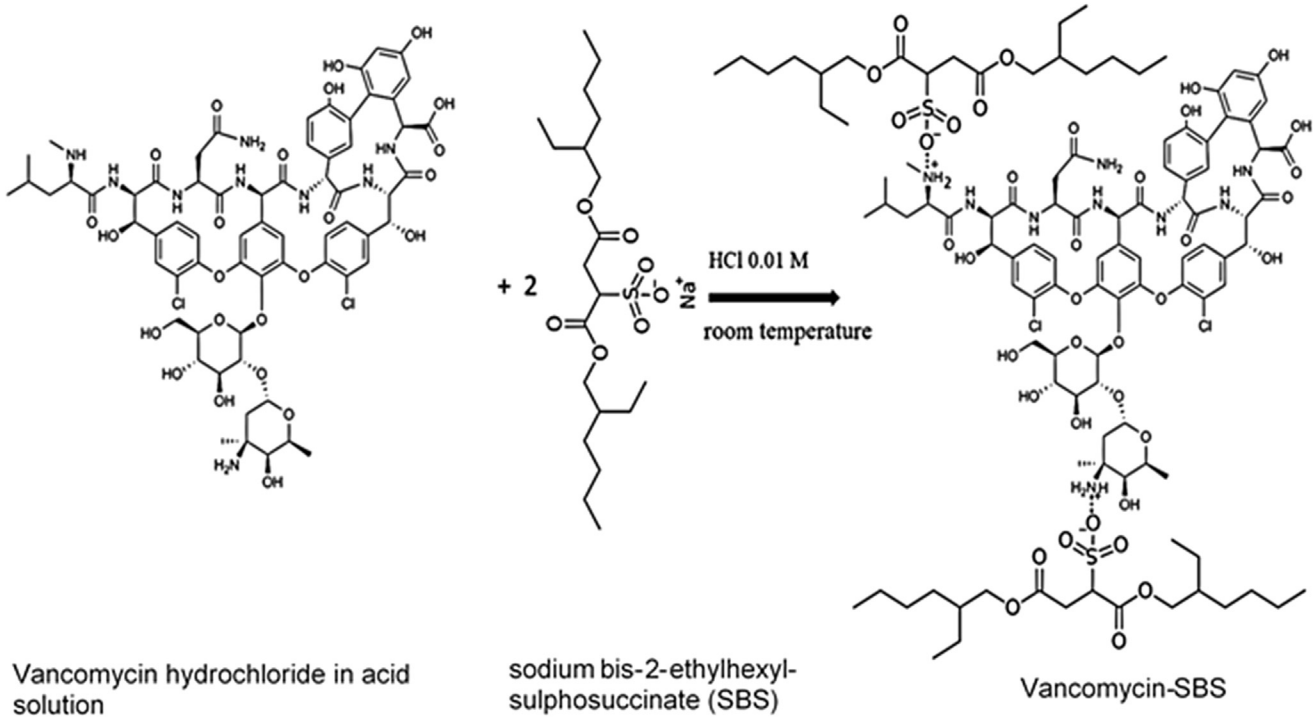


Figure 1. Reaction scheme between vancomycin and SBS resulting in the vancomycin-SBS complex (Van-SBS).

dispersion of FDA-labeled SEDDS formulations in 20 mM PBS pH 6.8 at a concentration of 1% were placed in one side of the tube containing 300 μL of mucus. Both sides of the tubes were covered with the stopper before set up on the rotor with horizontal rotation at the rate of 50 rpm in an incubator at 37°C for 4 h. Thereafter, the tubes were frozen at −80°C for 1 h followed by cutting into 10 slices of 2 mm length. The slices were numbered beginning from the donor side. FDA content in each tube slice was hydrolyzed using 400 μL of 5 M sodium hydroxide. The samples were incubated at 37°C for 30 min before centrifugation at 10,530 rcf. The fluorescence of supernatant was measured using microplate reader Tecan at an excitation and emission wavelength of 485 nm and 535 nm, respectively. As 100% control, the initial amount of applied FDA was measured as well. The percentage of permeated FDA versus this control was determined in each segment of mucus.

Statistical Analysis

Paired sample t-test was used to analyze the differences of droplet size and zeta potential between 0 h and 4 h of each SEDDS composition. One-way ANOVA followed by Tukey HSD test was used to compare droplet size, zeta potential, cell viability, vancomycin stability against papain, mobility in mucus, and percentage of permeation between all SEDDS compositions. All statistical analyses were performed using SPSS 17 at 95% confidence level ($p < 0.05$).

Results

Hydrophobic Ion Pairing

Interaction of the anionic groups of the surfactant SBS with the cationic substructures of vancomycin resulted in precipitation indicating the formation of HIP as shown in Figure 1. Surfactant to drug ratio played an important role in the yielded precipitate. Figure 2a indicates that low surfactant to drug ratio resulted in less HIP formation. Increasing the ratio yielded in more precipitate in case of both surfactants and reached highest precipitation at the ratio of 3:1. Using SDBS, the PE was decreased extensively at the ratio of 5:1 and dropped to zero at 7:1. In the case of SBS, a different behavior was observed. At the ratio of 5:1 and 7:1, no significant decrease in precipitation efficacy was observed. Figures 2b and 2c show the effect of surfactant to drug ratio on zeta potential of yielded HIP. The more of the anionic surfactant was added to the cationic drug, the more negative became the zeta potential of HIP showing that even more than 2 surfactant molecules can be, at least loosely, bound to 1 drug molecule.

FTIR Characterization of HIP

FTIR was applied to characterize the spectrum of vancomycin and surfactant modified vancomycin. As depicted in Figure 3, both SBS and SDBS showed CH and S=O stretching bands at about 2800 cm^{-1} and double separated peak at about 1150 cm^{-1} and about 1300 cm^{-1} wave number, respectively. The strong peak at about 1750 cm^{-1} (opened star) corresponds to C=O of ester as a characteristic peak of SBS over SDBS. The ortho-substituted aromatic ring of SDBS gave medium single peak at about 735 cm^{-1} (opened triangle).[34] Regarding vancomycin spectrum, similar to previously documented data,[35] a strong and board peak from 3200 to 3700 cm^{-1} (closed star) corresponds to O-H stretching of alcohol and carboxylic acid at the C terminus of the peptide. Such board peak decreased the available N-H stretching of amine group. Small peak at about 2800 cm^{-1} in vancomycin spectra came from short carbon as side chain of N-terminal amino acid. The precipitated product showed both vancomycin and surfactant characteristic bands, that is strong broadening peak of O-H stretching (closed star) and increasing the intensity of C-H stretching bands (closed triangle) in both products. A small shift of sulfonate separated peak is an indication that the interaction involved such functional groups.

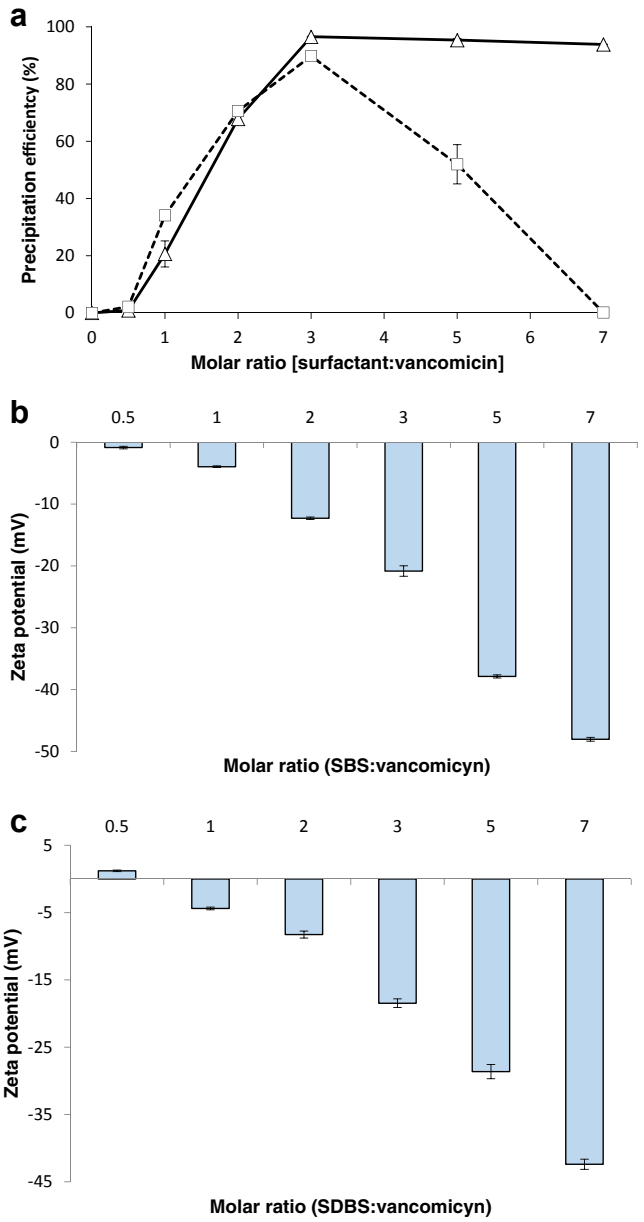


Figure 2. (a) Precipitation efficiency of HIP between vancomycin and sodium bis-2-ethylhexyl-sulphosuccinate (SBS: —△—) and HIP between vancomycin and sodium dodecylbenzene sulfonate (SDBS: ---□---), (b) change of zeta potential during HIP of vancomycin and SBS and (c) change of zeta potential during HIP of vancomycin and SDBS. The experiment was performed at indicated molar ratios between vancomycin and surfactants. Indicated values are means ±SD of 3 experiments.

Formulation and Characterization of SEDDS

SEDDS is mainly composed of oil as the inner phase of droplets after dispersion. To yield a high payload, solvents providing high solubility of HIP were chosen. Based on the solubility classification in European Pharmacopoeia, the produced vancomycin-SBS and vancomycin-SDBS ion pair are categorized to be very slightly soluble in oleic acid, oleum lini virginium, Captex 355, Capryol 90, Labrafil M 1944 CS, Miglyol 840, and SP Crodamol GTCC MBAL-LQ-(MV). In Capmul MCM, vancomycin-SDBS ion pair was also poorly soluble, whereas vancomycin-SBS ion pair exhibited high solubility of more than 25%.[36] Moreover, the log $P_{octanol/water}$ of Van-SBS was 1.4 unit higher than that of unmodified vancomycin. Therefore, Van-SBS was chosen for the development of SEDDS using Capmul MCM as solvent.

Optimization of SEDDS Composition

Capmul MCM, Kolliphor EL, and propylenglycol were used as solvent, surfactant, and co-solvent in SEDDS, respectively. The percentage of these excipients was optimized to generate isotropic mixtures exhibiting low droplet size once dispersed in 20 mM PBS pH 6.8 at a ratio of 1:100. As shown in Table 2, F1 and F2 displayed a droplet size much lower than F3, which is advantageous for their mucus permeating properties. F3 was also not chosen on account of its broad size distribution as indicated by PI > 0.5. Even though F1 displayed lower droplet size than F2, its low drug payload owing to low solvent content is a drawback. Therefore, F2 was chosen for further studies. Using this SEDDS formulation, the log $D_{SEDDS/release\ medium}$ value determined by Van-SBS solubility ratio in SEDDS to 20 mM PBS pH 6.8 was 2.2. The percentage of drug located in the droplets once SEDDS are dispersed can be calculated based on log D and volume ratio of SEDDS to dispersion medium.[31] Therefore, SEDDS droplets produced by dispersion of Van-SBS-SEDDS in 20 mM PBS pH 6.8 at a volume ratio of 1:100 contain 55.73% of total Van-SBS once equilibrium has been reached.

Characterization of Droplet Size and Zeta Potential

As shown in Figure 4, addition of Van-SBS did not change the size and zeta potential of generated SEDDS droplets. Both droplet size and zeta potential data indicated that the Van-SBS is located in the inner phase of droplets rather than anchored on the surface. This theory is also supported by the structure of Van-SBS as shown in Figure 1. Introduction of papain-palmitate decreased the droplet size as indicated in Figure 4a. The significant ($p < 0.05$) drop in zeta potential of SEDDS modified by papain-palmitate compared with blank SEDDS indicates that papain-palmitate assembles on the surface of the droplets (Fig. 4c). According to the results of physical stability studies, there was neither any phase separation nor precipitation of the SEDDS preconcentrate observed.

Vancomycin Stability Against Papain in SEDDS Dispersion

After dispersion using 20 mM PBS pH 6.8, papain-palmitate immobilized on SEDDS droplets did not hydrolyze vancomycin as shown in Figure 5. The concentration of vancomycin in Van-SBS-SEDDS containing papain-palmitate dispersion remained constant during 180 h of experiment similar to the dispersion of free enzyme Van-SBS-SEDDS and vancomycin solution containing papain-palmitate SEDDS droplets as control. Stability of vancomycin as well as Van-SBS against papain originates from the complexity of structure.

Cytotoxicity Study

Surfactants are well-known to be able to destroy cell membranes resulting in cell death.[37] Because Van-SBS as well as SEDDS contain surfactants, cytotoxicity studies were performed to address this issue. Figure 6 shows that the viability of Caco-2 cells after incubation with a medium containing SEDDS, Van-SBS-SEDDS, and Van-SBS-SEDDS containing papain-palmitate was significantly ($p < 0.05$) higher than the positive control. In the positive control, only 21% of cells survived, whereas all SEDDS formulations showed cell viability of more than 98%. According to ISO 10993-5:2009, the lowest limit of cell viability of nontoxic materials is 70%.[38] Therefore, all SEDDS formulations were nontoxic to cells indicating that none of the components in SEDDS damaged cells, including HIP and papain-palmitate.

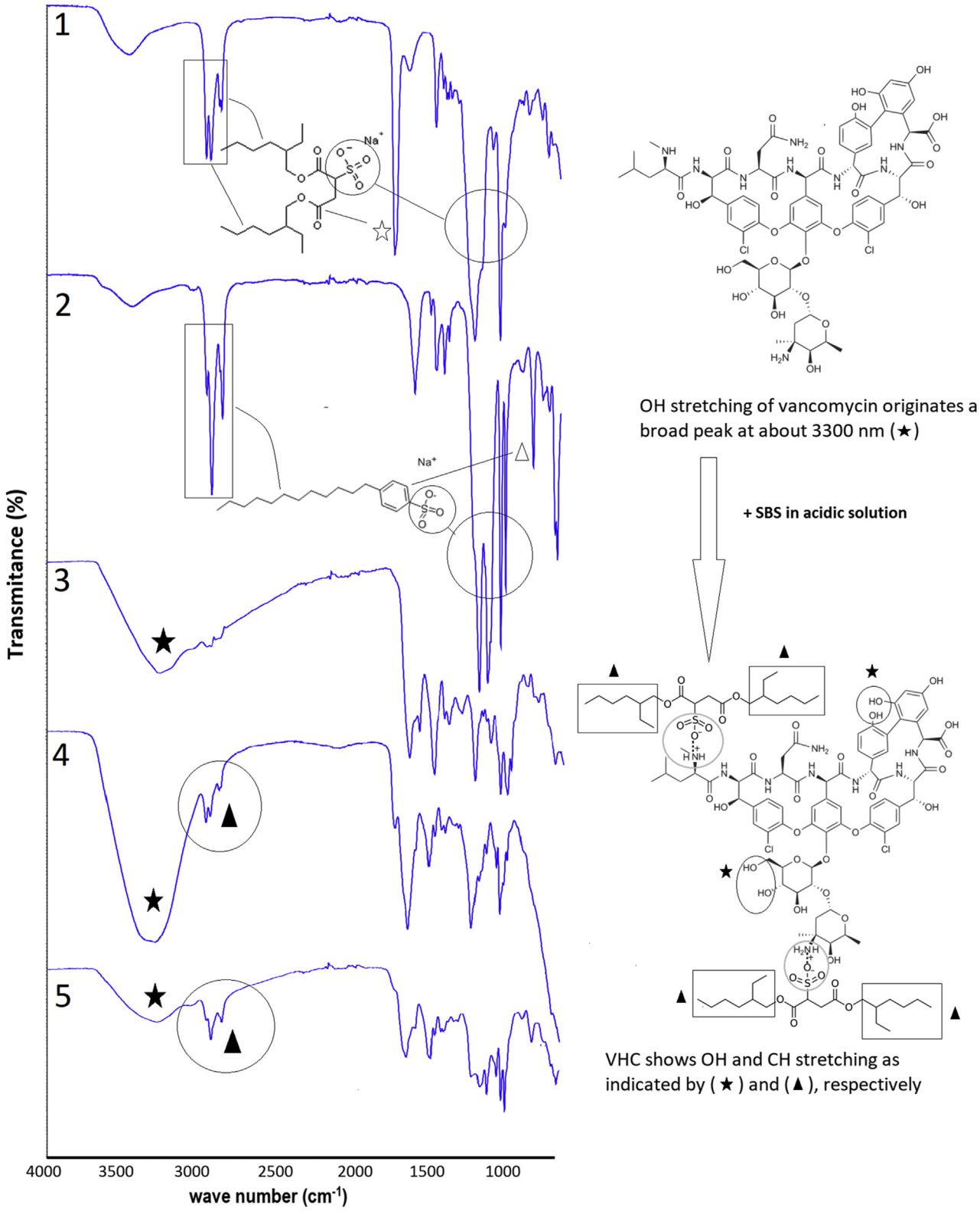


Figure 3. Fourier-transform infrared spectroscopy (*FTIR*) of sodium bis-2-ethylhexyl-sulphosuccinate (SBS) (1), sodium dodecylbenzene sulfonate (SDBS) (2), vancomycin hydrochloride (3), vancomycin-SBS complex (4), and vancomycin-SBDS complex (5).

Mucus Permeation Behavior of SEDDS

Transwell diffusion and rotating tube method were performed to determine the mucus permeability of SEDDS droplets containing peptide antibiotic. The diffusion studies of SEDDS droplets were performed using porcine intestinal mucus, and FDA was used as a marker as it can be easily incorporated in SEDDS. Figure 7a indicates that FDA-labeled blank SEDDS and FDA-labeled Van-SBS-SEDDS exhibit similar permeation explaining that Van-SBS did not affect the surface properties of droplets. SEDDS droplets

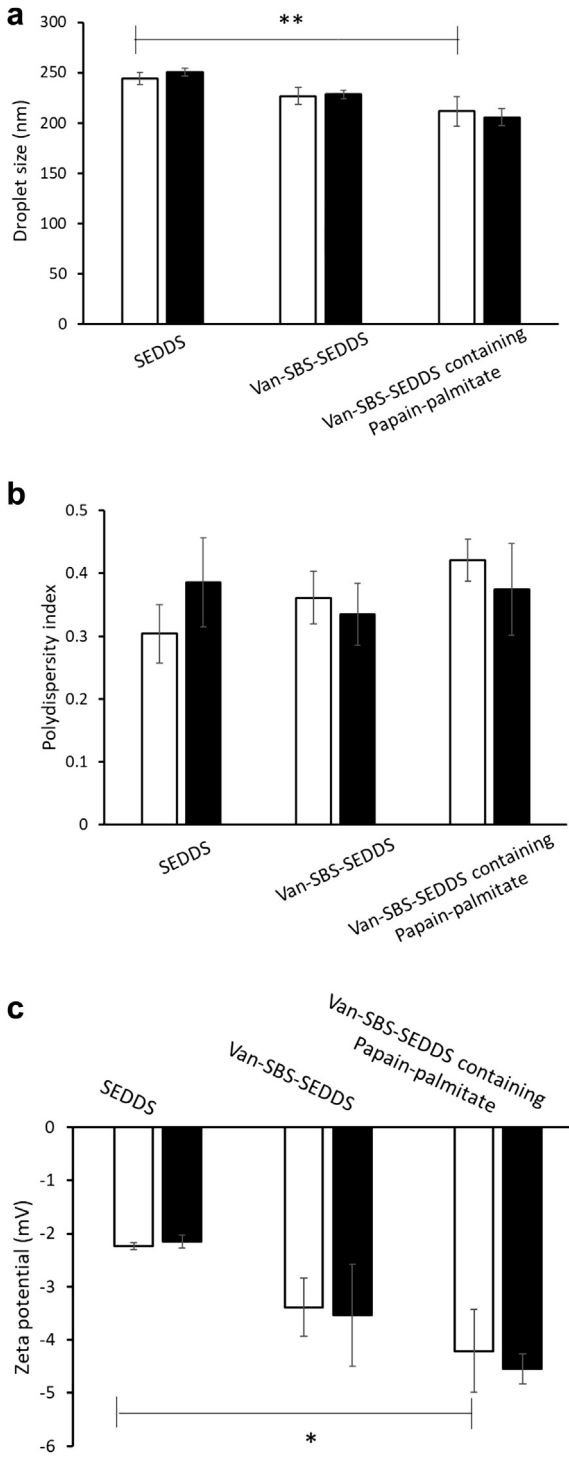


Figure 4. (a) Droplet size, (b) polydispersity index, and (c) zeta potential of SEDDS, 5% Van-SBS in SEDDS and Van-SBS-SEDDS containing papain-palmitate after having been diluted 1:100 in 20 mM PBS pH 6.8 at 0 (white bars) and 4 h (black bars). Indicated values are means ± SD of 3 experiments, $^{*}p < 0.05$, $^{**}p < 0.01$.

composition without proteolytic enzyme exhibited a permeation of around 9% within 4 h. The mucolytic activity of papain-palmitate in Van-SBS-SEDDS containing papain-palmitate resulted in a 2.8-fold higher droplets permeation in comparison to compositions without papain-palmitate. In contrast, vancomycin could not permeate the mucus at all within an observation period of 4 h (data not shown). It is likely that the polycationic peptide is entrapped in the polyanionic mucus.[24]

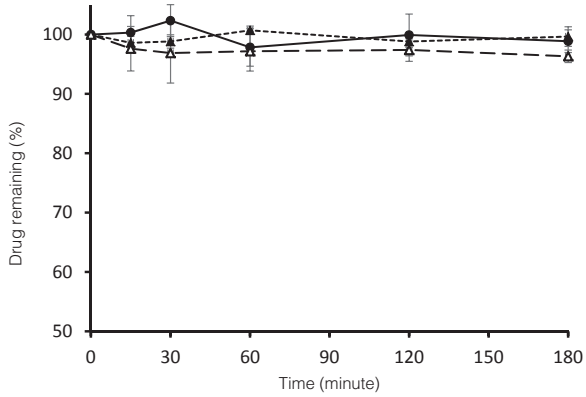


Figure 5. Vancomycin stability in Van-SBS-SEDDS containing papain-palmitate (closed triangle) after being diluted 1:100 in 20 mM PBS pH 6.8 in comparison to vancomycin solution in 20 mM PBS pH 6.8 containing papain-palmitate immobilized on SEDDS droplets (open triangle) and free enzyme SEDDS dispersion as control (closed circle). Indicated values are means ± SD of 3 experiments.

There are numerous ways to determine the capability of papain-palmitate to increase the mucus permeation of SEDDS droplets containing Van-SBS, such as the rotating tube method. As presented in Figure 7b, only droplets from SEDDS containing papain-palmitate penetrated mucus more than 27%. This value was 3.3-fold higher than that for SEDDS without papain-palmitate which was higher than the permeation enhancement observed in Transwell mucus diffusion studies.

Discussion

As peptide-based antibiotics can form numerous hydrogen bonds with mucus glycoproteins, their diffusion through the mucus gel layer is comparatively poor. Moreover, as most peptide-based antibiotics such as colistin, polymyxin B, bacitracin, and gramicidin S[7] exhibit a pronounced cationic net charge, they are immobilized in the 3-dimensional network of mucus owing to ionic interactions. On account of its glycosidic substructure and cationic nature, vancomycin is regarding its mucus permeation properties a challenging representative of this class of antibiotics. Strong interaction between the cationic vancomycin and negatively charged mucus[24] could be avoided by incorporating the drug into SEDDS as nanocarrier. Apart from ionic interactions, the viscoelastic properties of mucus represent a barrier decreasing the diffusion coefficient of macromolecule drugs and nanocarriers.[39] A rational

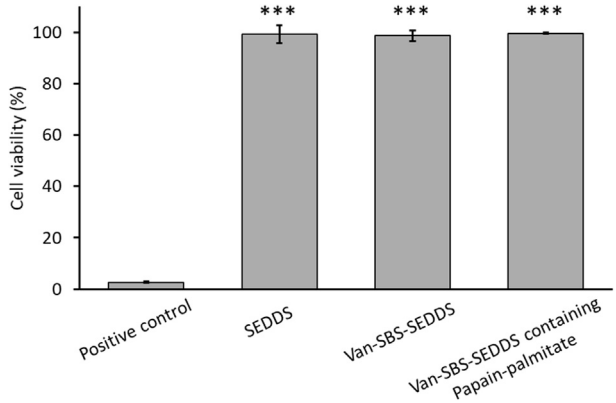


Figure 6. Cytotoxicity of blank SEDDS, Van-SBS-SEDDS, and Van-SBS-SEDDS containing papain-palmitate at a concentration of 0.1% dispersion in HBSS. Indicated values are means ± SD of 3 experiments, $^{***}p < 0.001$ in comparison to positive control.

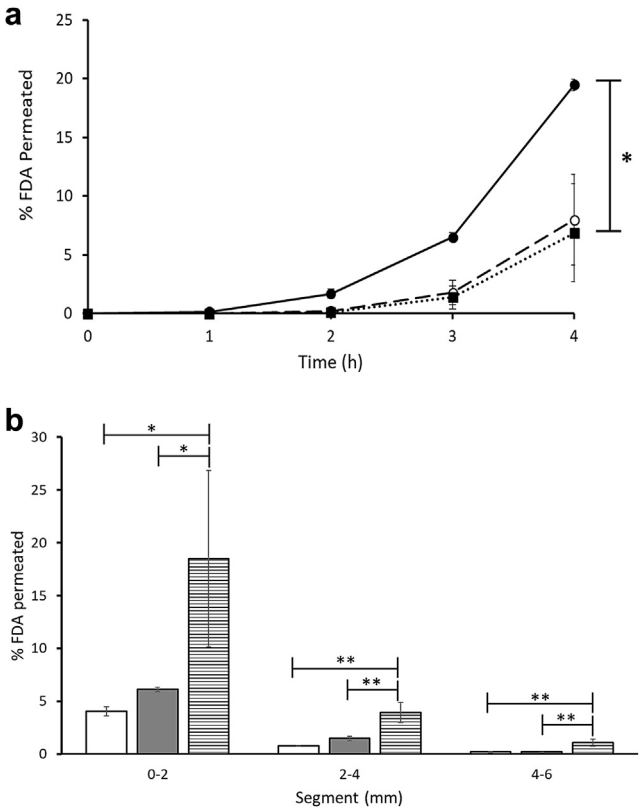


Figure 7. (a) FDA-labeled SEDDS (—○—), Van-SBS-SEDDS (··■··), and Van-SBS-SEDDS containing papain-palmitate (——●——) droplets permeation through mucus during 4 h experiment using Transwell diffusion, (b) FDA-labeled SEDDS (white bar), Van-SBS-SEDDS (gray bar), and Van-SBS-SEDDS containing papain-palmitate (horizontal line bar) droplets permeation during 4 h experiment using rotating tube method. Indicated values are means ± SD of 3 experiments, $^{*}p < 0.05$, $^{**}p < 0.01$.

approach to overcome the mucus gel layer covering the intestinal membrane is featuring papain-palmitate as mucolytic agent on SEDDS droplets. However, the hydrophilic nature of vancomycin hinders its compatibility with lipophilic components of SEDDS. To increase the miscibility of vancomycin with SEDDS components, the hydrophilicity of vancomycin was reduced by formation of HIP. The HIP reaction requires acidic conditions to ensure that the N amino group available in N-termini as well as the amino group on the heterocycle of vancomycin are protonated. Because vancomycin contains 2 cationic groups and SBS exhibits just 1 anionic group, the vancomycin molecule needs 2 surfactant molecules. Therefore, the maximum surfactant to drug ratio is expected to be 2:1. However, in present research, the maximum ratio of surfactant to drug was achieved at 3:1, which means that more surfactant with regard to the expected ratio is needed. An excess of surfactant is likely required to maintain the equilibrium between HIP and free vancomycin. Previous research using SBS resulted in the highest precipitation of HIP at a surfactant to drug ratio of 2:1, 6:1, and 3:2 for leuprorelin, insulin, and desmopressin, respectively,[30] correlating to the net positive charges of the respective peptide drug.

Above the maximum surfactant to drug ratio (3:1), in case of SDBS, PE was decreased as shown in Figure 2a. It is likely due to the formation of micelles by the excess amount of surfactant that could incorporate HIP avoiding precipitation.[40] The structure of surfactant determines the ability to form micelles. SDBS is a linear surfactant containing 1 lipophilic tail and 1 hydrophilic head group. Therefore, it can easier form micelles than SBS showing 2 lipophilic tails with the hydrophilic head group in between. As a result, the PE is decreasing intensively in the HIP formation using SDBS above the maximum surfactant to drug ratio, whereas SBS exhibited no significant decline.

In this study, SBS could be identified as powerful counterion for vancomycin to yield a lipophilicity enhancement resulting in solubility in Capmul MCM of up to 25%. Accordingly, 5% of drug payload in SEDDS containing 0.5% of papain-palmitate could be achieved. The position of the 2 attached lipophilic surfactants on the amino groups of vancomycin are obviously advantageous for Van-SBS to accumulate inside SEDDS droplets, as likely no polar head group remains. The incorporation of Van-SBS and papain-palmitate at these concentrations did not increase SEDDS droplets size. Even, the droplet size was decreased in the formulation containing papain-palmitate which is an advantage for permeation. This observation might be explained by the surfactant properties of acyl papain containing a lipophilic acyl group and a hydrophilic head group in form of papain.[41]

Regarding the stability of vancomycin against proteases, 1 might assume that the protease papain will hydrolyze the peptide drug vancomycin. Therefore, assessing the stability of vancomycin against papain-palmitate in present SEDDS formulation is a critical issue. Even though vancomycin shows stability against intestinal proteases,[25,26] exogenous proteases such as papain might hydrolyze vancomycin. Within this study, however, it was evidenced that vancomycin is not degraded by papain-palmitate. This stability of vancomycin is primarily provided by its chemical structure. The isobutyl group as a side chain of amino acid number 1 (AA-1), counted from N-termini, stabilizes its peptide bond with AA-2. This effect is similar to the acylation of N-termini providing a lipophilic nature of peptides increasing their stability.[42] Polyphenyl ether chain containing 3 aromatic rings connecting AA-2, AA-4, and AA-6 protects the peptide bond number 2-5, whereas peptide bond number 6 connecting AA-6 and AA-7 is protected by 3-hydroxy biphenyl group attaching as side chain of AA-5 and AA-7. The cyclic interconnection between amino acid employed by polyphenyl ether chain and hydroxy-biphenyl group mimics the formation of cyclic peptide drug which has been known to be stable.[43]

The chemically stable and nontoxic formulation of SEDDS was further evaluated regarding mucus permeation. The negative charge of generated droplets avoided intense ionic interactions with mucus[44] resulting in greater mobility. Nevertheless, SEDDS droplets showing higher size than drug molecules are to a higher extent obstructed by the 3-dimensional network of mucus. This dense network of mucins is an effective barrier for most macromolecules that directly correlates with their molecular weight referred to as steric barrier of mucus.[45] This obstruction resulted in a low mucus permeation of SEDDS droplets.

Introducing 0.5% papain-palmitate as mucolytic enzyme into SEDDS composition significantly increased mucus permeation. As cysteine protease bearing 3 active sites at amino acid number 158, 292 and 308,[46] papain shows the capability to breakdown the peptide bond of unglycosylated region of mucin, resulting in reduction of mucus viscosity.[47] Modified papain anchored on SEDDS droplets loosened the mucus resulting in permeation enhancement as evaluated using Transwell diffusion as well as rotating tube method. Comparing to another protease, this enhancement is higher than that obtained in previous research using trypsin HIP ion pair. At a protease concentration of 1%, an 2.6-fold enhancement was achieved.[28] Previous researches reported that surface modification of polyacrylic nanoparticles using bromelain and papain increased mucus permeation 2.5- and 3-fold, respectively, correlating with the results obtained in this study. However, the concentration of proteases in such polyacrylic nanoparticles were much higher, that is 21%-40%[19,33] in comparison to SEDDS droplets. The distribution of proteases in nanocarriers determines their permeation enhancing properties. In SEDDS formulation, the enzyme anchors on droplets surface as illustrated in Figure 8, whereas in nanoparticles, it is

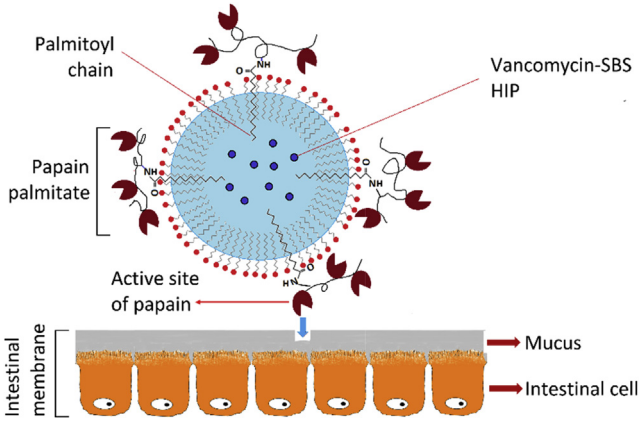


Figure 8. Schematic representation of the mucolytic effect of papain-palmitate anchored on surface SEDDS droplets containing vancomycin-SBS.

possible that the enzyme is also located to a high extent inside the particles as illustrated in previous research.[19] Accordingly, papain-palmitate–modified SEDDS are likely more efficient nanocarriers for oral drug delivery. SEDDS as nanocarrier is effective for both lipophilic and hydrophilic drugs. In the case of hydrophilic drugs such as peptide-based drugs, they should be lipidized for instance by HIP formation using surfactant, as in present research. Apart from peptide antibiotics, the carrier system described in this study should work also for peptide-based drugs in general as long as they are not enzymatically cleaved by papain.

The surface modification presented in this research complements the achievement of peptide drugs oral administration. Previously, co-delivery of peptide drugs with protease inhibitors, such as camostat and aprotinin, was developed to reduce catalytic hydrolysis in the intestinal tract.[48] However, peptide drugs which are not degraded by proteases, either owing to their original stable nature or protected by protease inhibitors still face other barriers including dense mucus network and intestinal membranes. Using synthetic peptides of the same molecular weight with different net charges, Li et al.[49] showed that synthetic anionic peptides rich in glutamic acid were less inhibited to diffuse in mucus than lysine-rich cationic peptides. Once the peptide drugs pass the mucus barrier, their hydrophilic properties lead them to follow the intercellular route avoiding the penetration through epithelial cells wrapped in a lipophilic membrane. Co-delivery of tight junction opening agents as absorption enhancers aids peptide absorption at this step.[50,51] Incorporation of peptide drugs in nanocarriers, such as nanoparticles[52] and niosomes,[53] provides both advantages: protecting the peptide and acting as an intestinal membrane permeation enhancer. However, the higher particle size of nanocarriers in comparison to molecular size of peptide increases the effectiveness of intestinal mucus steric barrier, which is surmounted by nanocarrier modification using protease.

In present research, for the first time, hydrophilic peptide-based antibiotic vancomycin was lipidized by HIP formation using anionic surfactant sodium bis-2-ethylhexyl-sulfosuccinate. Moreover, SEDDS droplets containing lipidized vancomycin were modified with papain-palmitate to overcome the low intestinal mucus permeation of vancomycin. The combination of vancomycin HIP and papain-palmitate in 1 SEDDS composition is a novel strategy for oral delivery of this peptide antibiotic.

Conclusion

Within current research, the improvement of intestinal mucus permeation of peptide antibiotic vancomycin could be achieved via incorporation into papain-palmitate–modified SEDDS as nanocarrier. Chemical modification of peptide antibiotic via formation of HIP between vancomycin and anionic surfactant resulting in sufficient solubility to be incorporated into SEDDS. Cytotoxicity study using Caco-2 cells indicated that all SEDDS formulations were nontoxic. Introduction of papain-palmitate along with Van-SBS-SEDDS did not hydrolyze vancomycin. The incorporation of papain-palmitate into SEDDS containing Van-SBS could overcome the intestinal mucus barrier owing to the proteolytic activity of papain resulting in a significant enhancement in SEDDS droplets permeation. It is evidenced in present research that introducing papain-palmitate at a concentration of 0.5% increased mucus permeation by 2.8-fold and 3.3-fold in the experiment using Transwell diffusion and rotating tube method, respectively. According to these results, featuring papain-palmitate into SEDDS containing Van-SBS can be a potential carrier to solve the problem of low mucus permeation of peptide drugs.

Statement Letter Regarding Access the Research Data

The research data used in preparation of the manuscript is available and can be accessed.

Acknowledgments

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References

1. Klein EY, Van Boeckel TP, Martinez EM, et al. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc Natl Acad Sci U S A*. 2018;115(15):E3463-E3470.
2. Antimicrobial Resistance. *The Global Risks Report*. Switzerland: The World Economic Forum in Geneva; 2018.
3. Munita JM, Arias CA. Mechanisms of antibiotic resistance. *Microbiol Spectr*. 2016;4(2):1-24.
4. Cosgrove SE. The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. *Clin Infect Dis*. 2006;42(Suppl 2):S82-S89.
5. Luther A, Bisang C, Obrecht D. Advances in Macrocyclic peptide-based antibiotics. *Bioorg Med Chem*. 2018;26(10):2850-2858.
6. Bechinger B, Gorr S-U. Antimicrobial peptides: mechanisms of action and resistance. *J Dent Res*. 2017;96(3):254-260.
7. Hancock REW, Chapple DS. Peptide antibiotics. *Antimicrob Agents Chemother*. 1999;43(6):1317-1323.
8. Paulus H, Gray E. The biosynthesis of polymyxin b by growing cultures of bacillus polymyxa. *J Biol Chem*. 1964;239:865-871.
9. Okuda K, Edwards GC, Winnick T. Biosynthesis of gramicidin and tryocidine in the dubos strain of Bacillus brevis. I. Experiments with growing cultures. *J Bacteriol*. 1963;85:329-338.
10. Shimura K, Sasaki T, Sugawara K. Biosynthesis of bacitracin, I. Formation of bacitracin by a subcellular fraction of bacillus licheniformis. *Biochim Biophys Acta*. 1964;86:46-55.
11. Lee KS, Lee BM, Ryu JH, Kim DH, Kim YH, Lim S-K. Increased vancomycin production by overexpression of MbtH-like protein in Amycolatopsis orientalis KFCC10990P. *Lett Appl Microbiol*. 2016;63(3):222-228.
12. Vara AG, Hochkoepple A, Nielsen J, Villadsen J. Production of teicoplanin by Actinoplanes teichomyceticus in continuous fermentation. *Biotechnol Bioeng*. 2002;77(5):589-598.
13. Talley K, Alexov E. On the PH-optimum of activity and stability of proteins. *Proteins*. 2010;78(12):2699-2706.
14. McClements DJ. Encapsulation, protection, and delivery of bioactive proteins and peptides using nanoparticle and Microparticle systems: a review. *Adv Colloid Interface Sci*. 2018;253:1-22.
15. Mahato RI, Narang AS, Thoma L, Miller DD. Emerging trends in oral delivery of peptide and protein drugs. *Crit Rev Ther Drug Carrier Syst*. 2003;20(2–3):153-214.
16. Richard J. Challenges in oral peptide delivery: lessons learnt from the clinic and future prospects. *Ther Deliv*. 2017;8(8):663-684.
17. Bhattacharjee S, Mahon E, Harrison SM, et al. Nanoparticle passage through porcine jejunal mucus: microfluidics and rheology. *Nanomedicine*. 2017;13(3):863-873.

18. Mansfield EDH, de la Rosa VR, Kowalczyk RM, et al. Side chain variations radically alter the diffusion of poly(2-Alkyl-2-oxazoline) functionalised nanoparticles through a Mucosal barrier. *Biomater Sci*. 2016;4(9):1318-1327.
19. Müller C, Perera G, König V, Bernkop-Schnürch A. Development and in vivo evaluation of papain-functionalized nanoparticles. *Eur J Pharm Biopharm*. 2014;87(1):125-131.
20. Liu Y, Breukink E. The membrane steps of bacterial cell wall synthesis as antibiotic targets. *Antibiotics*. 2016;5(3):1-22.
21. Wang F, Zhou H, Olademehin OP, Kim SJ, Tao P. Insights into key interactions between vancomycin and bacterial cell wall structures. *ACS Omega*. 2018;3(1):37-45.
22. Czepiel J, Dróżdż M, Pituch H, et al. Clostridium difficile infection: review. *Eur J Clin Microbiol Infect Dis*. 2019. https://doi.org/10.1007/s10096-019-03539-6 [e-pub ahead of print].
23. Marconescu P, Graviss EA, Musher DM. Rates of killing of methicillin-resistant Staphylococcus Aureus by ceftaroline, daptomycin, and telavancin compared to that of vancomycin. *Scand J Infect Dis*. 2012;44(8):620-622.
24. Bernkop-Schnürch A. Strategies to overcome the polycation dilemma in drug delivery. *Adv Drug Deliv Rev*. 2018;136-137:62-72.
25. McHenry MC, Gavan TL. Vancomycin. *Pediatr Clin North Am*. 1983;30(1):31-47.
26. Jordan DC, Reynolds PE. Vancomycin. In: Mechanism of Action*; Antibiotics*. Berlin, Heidelberg: Springer; 1967:102-116.
27. Fukushima K, Okada A, Hayashi Y, et al. Enhanced oral bioavailability of vancomycin in rats treated with long-term parenteral nutrition. *Springerplus*. 2015;4:442.
28. Shahzadi I, Dizdarević A, Efiana NA, Matuszczak B, Bernkop-Schnürch A. Trypsin decorated self-emulsifying drug delivery systems (SEDDS): key to enhanced mucus permeation. *J Colloid Interface Sci*. 2018;531:253-260.
29. Xue Y, Wu C-Y, Branford-White CJ, Ning X, Nie H-L, Zhu L-M. Chemical modification of stem bromelain with Anhydride groups to enhance its stability and catalytic activity. *J Mol Catal B Enzym*. 2010;63(3):188-193.
30. Griesser J, Hetényi G, Moser M, Demarne F, Jannin V, Bernkop-Schnürch A. Hydrophobic ion pairing: key to highly payloaded self-emulsifying peptide drug delivery systems. *Int J Pharm*. 2017;520(1−2):267-274.
31. Bernkop-Schnürch A, Jalil A. Do drug release studies from SEDDS make any sense? *J Control Release*. 2018;271:55-59.
32. Friedl H, Dünnhaupt S, Hintzen F, et al. Development and evaluation of a novel mucus diffusion test system approved by self-nanoemulsifying drug delivery systems. *J Pharm Sci*. 2013;102(12):4406-4413.
33. Pereira de Sousa I, Cattoz B, Wilcox MD, et al. Nanoparticles decorated with proteolytic enzymes, a promising strategy to overcome the mucus barrier. *Eur J Pharm Biopharm*. 2015;97(Pt A):257-264.
34. Stuart B. *Infra Red Spectroscopy: Fundamentals and Applications*. Hoboken: John Wiley & Sons; 2004.
35. Zarif MS, Afidah AR, Abdullah JM, Shariza AR. Physicochemical Characterization of Vancomycin and its Complexes with SS-Cyclodextrin. *Biomed Res (India)*. 2012;23(4):513-520.
36. Council of Experts and its Expert Committees of United State Pharmacopeial Convention. *The United States Pharmacopeia 38*. 4/a. Rockville: The United States Pharmacopeial Convention; 2015.
37. Aránzazu Partearroyo M, Ostolaza H, Goñi FM, Barberá-Guillem E. Surfactant-induced cell toxicity and cell lysis. A study using B16 Melanoma cells. *Biochem Pharmacol*. 1990;40(6):1323-1328.
38. ISO. ISO 10993-5:2009, Biological evaluation of medical devices – Part 5: tests for in vitro cytotoxicity. 2009. Available at: http://www.iso.org/cms/render/live/en/sites/isoorg/contents/data/standard/03/64/36406.html. Accessed May 2, 2019.
39. Celli JP, Turner BS, Afdhal NH, et al. Rheology of gastric mucin exhibits a PH-dependent sol-gel transition. *Biomacromolecules*. 2007;8(5):1580-1586.
40. Torchilin VP. Micellar nanocarriers: pharmaceutical perspectives. *Pharm Res*. 2007;24(1):1-16.
41. Dexter AF, Middelberg APJ. Peptides as functional surfactants. *Ind Eng Chem Res*. 2008;47(17):6391-6398.
42. Patel G, Misra A. 10 - oral delivery of proteins and peptides: concepts and applications. In: Misra A, ed. *Challenges in Delivery of Therapeutic Genomics and Proteomics*. London: Elsevier; 2011:481-529.
43. Hess S, Ovadia O, Shalev DE, et al. Effect of structural and conformation modifications, including backbone cyclization, of hydrophilic hexapeptides on their intestinal permeability and enzymatic stability. *J Med Chem*. 2007;50(24):6201-6211.
44. Korytowski A, Abuillan W, Amadei F, et al. Accumulation of phosphatidylcholine on gut mucosal surface is not dominated by electrostatic interactions. *Biochim Biophys Acta Biomembr*. 2017;1859(5):959-965.
45. Liu M, Zhang J, Shan W, Huang Y. Developments of mucus penetrating nanoparticles. *Asian J Pharm Sci*. 2015;10(4):275-282.
46. Papain precursor - Carica papaya (Papaya). Available at: http://www.uniprot.org/uniprot/P00784. Accessed April 6, 2018.
47. Müller C, Leithner K, Hauptstein S, Hintzen F, Salvenmoser W, Bernkop-Schnürch A. Preparation and characterization of mucus-penetrating papain/poly(Acrylic acid) nanoparticles for oral drug delivery applications. *J Nanoparticle Res*. 2013;15(1):1353.
48. Tozaki H, Emi Y, Horisaka E, Fujita T, Yamamoto A, Muranishi S. Degradation of insulin and calcitonin and their protection by various protease inhibitors in rat caecal contents: implications in peptide delivery to the colon. *J Pharm Pharmacol*. 1997;49(2):164-168.
49. Li LD, Crouzier T, Sarkar A, Dunphy L, Han J, Ribbeck K. Spatial configuration and composition of charge modulates transport into a mucin hydrogel barrier. *Biophys J*. 2013;105(6):1357.
50. Tscheik C, Blasig IE, Winkler L. Trends in drug delivery through tissue barriers containing tight junctions. *Tissue Barriers*. 2013;1(2):e24565.
51. Bocsik A, Walter FR, Gyebrovszki A, et al. Reversible opening of intercellular junctions of intestinal epithelial and brain endothelial cells with tight junction Modulator peptides. *J Pharm Sci*. 2016;105(2):754-765.
52. Sharma G, Sharma AR, Nam J-S, Doss GPC, Lee S-S, Chakraborty C. Nanoparticle based insulin delivery system: the next generation efficient therapy for type 1 diabetes. *J Nanobiotechnology*. 2015;13:74.
53. Chauhan MK, Bhatt N. Bioavailability enhancement of polymyxin B with novel drug delivery: development and optimization using quality-by-design approach. *J Pharm Sci*. 2019;108(4):1521-1528.