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Self-nanoemulsifying drug delivery systems (SNEDDS) for oral delivery of protein drugs
II. *In vitro* transport study

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ABSTRACT

To develop a self-nanoemulsifying drug delivery system (SNEDDS) for protein drugs, and particularly, to test the *in vitro* transport of β-lactamase (BLM) by SNEDDS across the cell monolayer. Fluorescently labeled BLM (FITC-BLM), a model protein, formulated into 16 SNEDDS preparations through a solid dispersion technique were studied for transport across MDCK monolayer. All the SNEDDS nanoemulsions resulted in higher transport rate than the free solution. The transport rate by SNEDDS depends on the SNEDDS composition. SNEDDS NE-12-7 (oil: Lauroglycol FCC, surfactant: Cremophor EL and a cosurfactant: Transcutol HP) at the ratio of 5:4:3, rendered the highest transportation rate, 33% as compared to negligible transport by the free solution. FITC-BLM solution mixed with the surfactant and the cosurfactant of SNEDDS NE-12-7 or with blank SNEDDS NE-12-7 increased the transport only by 3.3 and 1.5 folds, respectively, compared to free solution alone. It was found that the monolayer integrity was not compromised in the presence of SNEDDS NE-12-7 or its surfactant/cosurfactant. The SNEDDS significantly increased the transport of FITC-BLM across MDCK monolayer *in vitro*. SNEDDS may be a potential effective delivery system for non-invasive protein drug delivery.

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1. Introduction

Proteins/peptides (hereafter called as proteins) are delivered usually by injections which are very inconvenient, painful and unsafe. Although non-invasive delivery routes such as oral administration for proteins is ideal, poor absorption because of low permeation of the protein due to its large size and hydrophilicity and extensive degradation of proteins are encountered in the harsh environment of the gastro-intestinal tract (GIT) when administered orally (Toorisaka et al., 2005; Almeida and Souto, 2007). In the GIT, the oils are absorbed effectively through various lipid absorption mechanisms such as pinocytosis, passive diffusion or endocytosis, or absorption through lymphatic system (Georgakopoulos et al., 1992). Thus, the hypothesis of the present research is that the proteins loaded inside oil may be effectively absorbed when the oil is absorbed. Therefore, the present goal was to develop such a delivery system in the form of nanoemulsion.

Nanoemulsions (also called as microemulsions in many previous publications) are thermodynamically stable system with the droplet size usually less than 100 nm. Nanoemulsions have been studied for oral delivery of proteins wherein the protein is dissolved in the aqueous phase of either an O/W or W/O nanoemulsion. The presence of protein in the aqueous phase may lead to long term stability problems and significant loss of the protein to the environmental aqueous media *in vivo*. Previous study showed the increase of oral bioavailability is very minimum (Cilek et al., 2005). Hence the present study aims to develop an O/W nanoemulsion in which the protein is loaded in the oil phase.

In our previous study (Venkata Ramana Rao and Shao, in press), a self-nanoemulsifying drug delivery system (SNEDDS) was used as a delivery system for β-lactamase (BLM), a 29 kDa model hydrophilic protein. SNEDDS is an isotropic mixture of oil, surfactant, and cosurfactant which upon dilution with aqueous media spontaneously form fine O/W nanoemulsion with less than 50 nm in droplet size (Nazzal et al., 2002). In that study (Venkata Ramana Rao and Shao, in press), it was shown that BLM was successfully loaded in SNEDDS via solid dispersion technique. Sixteen different prototypes of BLM-loaded

Abbreviations: BLM, β-lactamase; DPBS, Dulbecco's phosphate-buffer saline; FITC, fluorescein isothiocyanate isomer I; MDCK, Madin-Darby canine kidney; DMEM, supplemented Dulbecco's modified Eagle's medium; SNEDDS, self-nanoemulsifying drug delivery system; TEER, trans-epithelial electrical resistance.

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Table 1
Composition of the 16 different SNEDDS

SNEDDS prototypes	Composition (O/SA/CoSA)	Ratio of O/SA/CoSA
NE-12-5	Lauroglycol FCC/Cremophor EL/Transcutol HP	3:2:1
NE-12-7	Lauroglycol FCC/Cremophor EL/Transcutol HP	5:4:3
NE-13-2	Lauroglycol FCC/Cremophor RH 40/Transcutol HP	4:3:2
NE-13-3	Lauroglycol FCC/Cremophor RH 40/Transcutol HP	5:4:1
NE-13-7	Lauroglycol FCC/Cremophor RH 40/Transcutol HP	5:4:3
NE-13 – 8	Lauroglycol FCC/Cremophor RH 40/Transcutol HP	3:4:3
NE-13-11	Lauroglycol FCC/Cremophor RH 40/Transcutol HP	3:4:1
NE-17-2	Caproyl-90/Cremophor EL/Transcutol HP	4:3:2
NE-17-3	Caproyl-90/Cremophor EL/Transcutol HP	5:4:1
NE-17-5	Caproyl-90/Cremophor EL/Transcutol HP	3:2:1
NE-17-7	Caproyl-90/Cremophor EL/Transcutol HP	5:4:3
NE-18-8	Caproyl-90/Cremophor RH 40/Transcutol HP	3:4:3
NE-37-5	Caproyl-90/Cremophor EL/PEG-400	3:2:1
NE-57-2	Caproyl-90/Cremophor EL/Propylene glycol	4:3:2
NE-57-3	Caproyl-90/Cremophor EL/Propylene glycol	5:4:1
NE-57-7	Caproyl-90/Cremophor EL/Propylene glycol	5:4:3

SNEDDS were capable of dissolving BLM dispersion followed by spontaneous formation of nanoemulsion with the addition of water. The present study was conducted to evaluate the transport of BLM by these sixteen different BLM-loaded SNEDDS prototypes *in vitro* across a biological membrane, MDCK cell monolayer.

MDCK (Madin-Darby canine kidney) cells have been shown to differentiate into columnar epithelium and to form tight junctions when cultured on semi-permeable membranes (Misfeldt et al., 1976; Cereijido et al., 1978). Similar to Caco-2 cells, MDCK cells have been used as a model cellular barrier for assessing intestinal epithelial drug transport (Simmons, 1982; Horio et al., 1989; Irvine et al., 1999; Taub et al., 2002). A study comparing the Caco-2 and MDCK cells (Irvine et al., 1999) indicated that the P_{app} values obtained from MDCK monolayers were in fact slightly more predictive of *in vivo* human intestinal absorption than those obtained from Caco-2. In addition, compared to Caco-2 cells, MDCK monolayers exhibit other advantages such as good morphological homogeneity that leads to good inter-laboratory reproducibility, and significantly shorter growth time in cell culture and thus less susceptible to contamination. Hence, in this present study, MDCK was used as the model cell line for cellular transport studies of BLM-loaded SNEDDS. The subsequent article reports the *in vivo* oral absorption studies of BLM-loaded SNEDDS.

2. Materials and methods

2.1. Materials

MDCK canine kidney cell line was purchased from ATCC (Rockville; MD, USA). Dulbecco's modified Eagle medium (DMEM), Dulbecco's phosphate-buffer saline solution, pH 7.4 (DPBS), 0.25% trypsin with 0.2 g/l ethylenediamine tetra acetic acid (EDTA), sodium pyruvate 100 mM solution, non-essential amino acids (100×) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA); β-lactamase Type II lyophilized powder (29 kDa; 1.26 mU/μg; from Bacillus cereus EC 3.5.2.6), DL-propranolol-[4-^{3}H] hydrochloride, D-mannitol-[1-^{3}H(N)] hydrochloride, fluorescein isothiocyanate isomer I (FITC), Fluorotag kit, propylene glycol (PG) and polyethylene glycol-400 (PEG-400) and other reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Free samples of Caproyl-90®, Transcutol HP® and Lauroglycol FCC® were supplied by Gattefosse Corp. (Paramus, NJ). Free samples of Cremophor EL® and Cremophor RH40® were provided by BASF Corp. (Florham Park, NJ, USA). Hydrogenated soy phosphatidyl choline (SoyPC) was purchased from Avanti Polar Lipid (Alabaster, AL, USA).

2.2. Preparation of fluorescent labeled BLM (FITC-BLM) SNEDDS formulations

BLM was fluorescently labeled with fluorescein isothiocyanate isomer I using the Fluorotag kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Detailed description of the formulation preparations of FITC-BLM-loaded SNEDDS is described in our previous paper (Venkata Ramana Rao and Shao, in press). Briefly, an aqueous dispersion of SoyPC in DPBS was mixed with FITC-BLM solution in DPBS in the ratio of 4:1 (SoyPC:BLM). Then this mixture was lyophilized to obtain the solid dispersion of FITC-BLM in SoyPC. Total 16 SNEDDS of various components were successfully prepared (Table 1). Then FITC-BLM-Soy PC dispersion was loaded into these SNEDDS (2 mg dispersion in 1 g of SNEDDS). The amount of BLM present in these SNEDDS was 2000 mU/g. The corresponding nanoemulsions were obtained by the addition of DPBS to the FITC-BLM-loaded SNEDDS at 2:1 ratios under gentle stirring (10 rpm). The droplet size of all these nanoemulsions were less than 50 nm.

2.3. Transport studies

2.3.1. Culture of MDCK cells in transwells

MDCK cells were cultivated in 75 cm^2 flasks in s-DMEM (Dulbecco's Modified Eagle's medium supplemented with sodium pyruvate solution, non-essential amino acids, and fetal bovine serum) at 37 °C, 95% air and 5% CO_2. The cells were harvested by the treatment of 0.25% trypsin with EDTA, seeded onto the polycarbonate filters of the transwells at a density of 0.3×10^5 cells/cm^2, and were further incubated under normal cell culture conditions. The growth media was replaced every other day and the trans-epithelial electrical resistance (TEER) measurement was monitored periodically by using a Millicell® ERS meter connected to a pair of chopstick Ag/AgCl electrodes. The monolayer, which became confluent after about 4–6 days when TEER reading reached a plateau, was used in the transport studies.

2.3.2. Transport of BLM through the MDCK monolayer

Prior to initiating BLM transport studies, the monolayers grown on the transwell inserts were washed with DPBS. FITC-BLM-loaded SNEDDS nanoemulsion (1.5 ml) was placed in the donor compartment. The media in the receiver chamber was replaced by 2.6 ml of DPBS. Sample (100 μl) was withdrawn from the receiver compartment at predetermined time points and replaced with fresh DPBS. The samples were assayed for FITC-BLM. The experiments were carried out in triplicate. First, the 16 different FITC-BLM-loaded SNEDDS nanoemulsions were screened for the highest transport

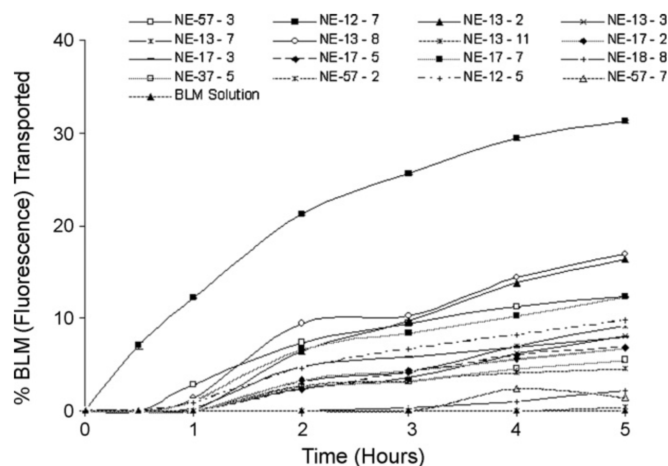

Fig. 1. Transport of BLM in the 16 BLM-loaded SNEDDS nanoemulsions through MDCK monolayers. FITC-BLM-loaded nanoemulsions or FITC-BLM free solution was added into the donor side of the confluent MDCK monolayer. Samples were withdrawn from the receiver side at different time points, and the transported FITC-BLM was assayed (mean ± S.D., $n = 3$).

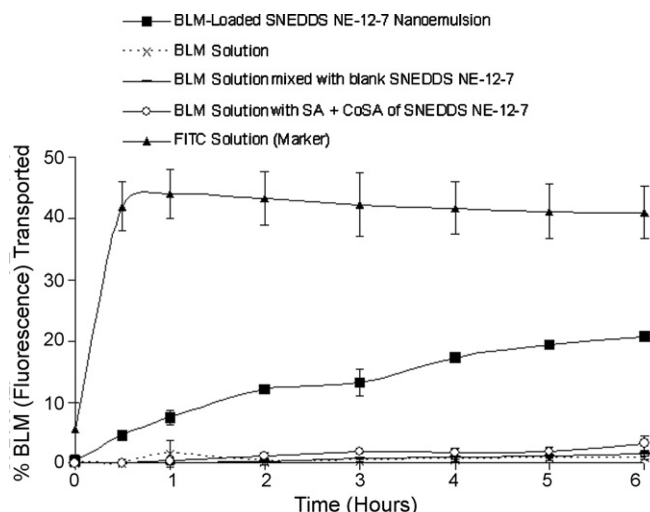

Fig. 2. Transport of BLM across MDCK monolayer in NE-12-7 nanoemulsion and various controls. To the donor side of the confluent MDCK monolayers, FITC-BLM was added in the form of NE-12-7 nanoemulsion, free solution, free solution mixed with blank NE-12-7 nanoemulsion, free solution mixed with the surfactant and cosurfactant of NE-12-7. FITC free solution was also used as one of the controls. Samples were withdrawn from the receiver side at different time points and assayed for fluorescence (mean ± S.D., $n = 3$).

through MDCK monolayer (passage # 62) and compared against FITC-BLM free solution. SNEDDS NE-12-7 nanoemulsion which showed highest transport was then further evaluated against various controls across the monolayer (passage # 64). The controls used were FITC-BLM free solution (made with DPBS); FITC-BLM solution mixed with blank SNEDDS NE-12-7 (2:1 ratio, nanoemulsion was formed and FITC-BLM was in the aqueous phase), FITC-BLM solution mixed with the surfactant and the cosurfactant of SNEDDS NE-12-7, and FITC free solution. In addition to the measurement of BLM at the receiver side, the droplet size at both sides were measured at 0, 2, 4 and 6 h by a Nicomp 380 ZLS laser diffraction sizer (PSS Nicomp, Santa Barbara CA, USA).

Regression analysis of the concentration of BLM in the receiver compartment vs. time was plotted and the slope of the regression line dc/dt was used to calculate P_{app}, apparent permeability coefficient.

$$P_{\mathrm{app}} = \frac{(\mathrm{d}c/\mathrm{d}t)}{A} \times C_0$$

where A is the surface area (cm^2) of the cell monolayer and C_0 is the initial concentration (mU/ml) in the donor compartment.

2.4. Effect of SNEDDS on the integrity of the MDCK monolayer

DL-Propranolol-[4-^{3}H] hydrochloride and D-mannitol-[1-^{3}H (N)] hydrochloride were used as paracellular and transcellular markers, respectively. Confluent MDCK monolayers grown in transwells were rinsed twice with DPBS. Then 2.6 ml of DPBS was added to the receiver compartment. Into the donor side, 1.5 ml of DPBS, or DPBS mixed with SNEDDS NE-12-7 (2:1 ratio), or DPBS mixed with the surfactant and the cosurfactant of SNEDDS NE-12-7 was added, which contained either 34.5 nM DL-propranolol-[4-^{3}H] hydrochloride or 66.6 nM D-mannitol-[1-^{3}H(N)] hydrochloride. The monolayers were further incubated for 6 h. At each time interval, 100 μl of sample was withdrawn from the receiver side and replaced with 100 μl of DPBS. At the 6th hour, 100 μl of sample was also withdrawn from the donor side. The samples were assayed for propranolol or mannitol by radioactive assay. TEER was monitored before, during and after the experiment to gauge the integrity of the monolayers.

2.5. Assay of FITC-BLM

FITC-BLM was assayed by a spectro-fluorimeter at excitation and emission wavelength of 485 and 535 nm, respectively, according to a standard curve method.

2.6. Radioactive assay of mannitol and propranolol

Samples (100 μl) containing the radio-labeled mannitol or propranolol was placed in the scintillation vials, and 10 ml of scintillation cocktail (Sigma) was added. These samples were counted for total counts per minute (cpm) on a Packard Tricarb LSC (PerkinElmer, Waltham, MA, USA). The cpm count was converted to the concentration according to a standard curve method.

2.7. Data analysis

ANOVA was used to compare the difference between the data of interest. Wherever possible, the data is presented as mean ± standard deviation.

3. Results

3.1. Transport of FITC-BLM through MDCK monolayers

The cumulative % FITC-BLM transported through the MDCK monolayer from the 16 BLM-loaded SNEDDS formulations is shown in Fig. 1. Based on the results, it was clearly seen that all the SNEDDS nanoemulsions showed significantly ($p < 0.05$) higher transport of FITC-BLM across the MDCK monolayer than the FITC-BLM solution. SNEDDS NE-12-7 nanoemulsion showed 33.1% cumulative transport of FITC-BLM at 5 h, the highest transport than any other SNEDDS, while the FITC-BLM solution rendered negligible transport at 5 h.

The results of the FITC-BLM transported from SNEDDS NE-12-7 nanoemulsion against various controls, free FITC solution (fluorescence marker), FITC-BLM solution, FITC-BLM solution mixed with blank SNEDDS NE-12-7, FITC-BLM solution mixed with the surfactant and the cosurfactant of NE-12-7 are shown in Fig. 2. The results

Table 2
Permeability coefficient ($P_{app} \times 10^7$, cm/s) of BLM in various preparations across the MDCK monolayers (mean ± S.D., $n = 3$)

Group	Description	Type	Permeability coefficient ($P_{app} \times 10^7$, cm/s)
1	BLM solution in DPBS	Solution	6.08 ± 2.79[b]
2	BLM-loaded NE-12-7 nanoemulsion	Nanoemulsion (BLM in oil phase)	240.97 ± 6.79[a]
3	BLM solution mixed with blank NE-12-7 SNEDDS	Nanoemulsion (BLM in aqueous phase)	20.53 ± 5.56[b]
4	BLM solution mixed with SA and CoSA of NE-12-7 SNEDDS	Micellar	38.54 ± 5.98[a,b]

[a] Significant difference from BLM solution ($p < 0.01$).
[b] Significant difference from BLM-loaded NE-12-7 nanoemulsion ($p < 0.01$).

indicate that the cumulative FITC-BLM transported from FITC-BLM-loaded SNEDDS NE-12-7 nanoemulsion was 24.9-fold greater than that by free solution alone ($p < 0.01$), whereas the transport from FITC-BLM solution mixed with the surfactant and the cosurfactant of SNEDDS NE-12-7 or with blank SNEDDS NE-12-7 as compared to free solution alone was not statistically significant ($p > 0.05$). The permeability values of FITC-BLM from various preparations are shown in Table 2. The permeability values indicate that the permeation of FITC-BLM from the SNEDDS NE-12-7 nanoemulsion was significantly higher than that from FITC-BLM solution or FITC-BLM solution mixed with surfactant and cosurfactant of SNEDDS NE-12-7 ($p < 0.01$). The results also indicate that free FITC solution showed the highest transport across the monolayer.

In order to investigate whether oil droplets transported across the monolayer during the transport study, the droplet size at the receiver and donor side was measured, and the results are shown in Table 3. It can be seen that at beginning (time 0) all the receiver sides had no droplets, but at 2, 4 and 6 h, the droplet size of the receiver side was almost the same as that at the respective donor side in all the cases. These results clearly indicate that the oil droplets of the o/w nanoemulsion were transported through the cell monolayer.

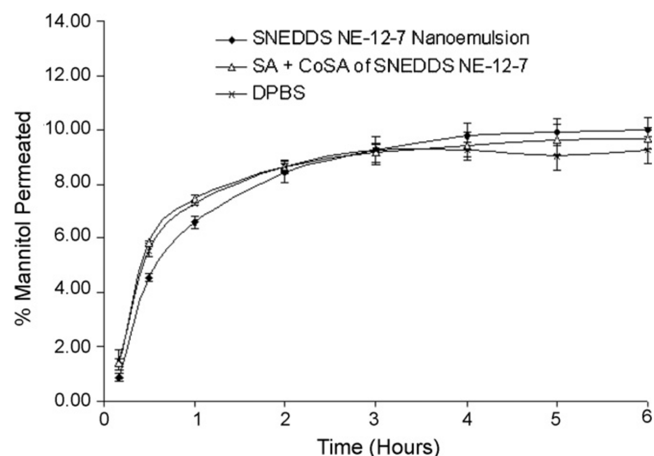

Fig. 3. Effect of SNEDDS NE-12-7 nanoemulsion and its surfactant/cosurfactant on the transport of mannitol through MDCK monolayer. To the donor side of the confluent MDCK monolayer, radio-labeled mannitol was added with or without NE-12-7 nanoemulsion or surfactant/cosurfactant of NE-12-7. Samples were withdrawn from the receiver side at different time points for the measurement of mannitol (mean ± S.D., $n = 3$).

3.2. *Effect of SNEDDS on the integrity of the MDCK monolayer*

The potential effect of SNEDDS on the MDCK monolayer integrity during the transport studies was examined by both a paracellular and a transcellular marker, mannitol and propranolol, respectively. Both the markers were added (with or without SNEDDS nanoemulsion, or its surfactant/cosurfactant) onto the monolayers and the transport of the markers across the monolayer was then monitored.

Fig. 3 shows the transport of mannitol across the MDCK monolayer in the presence of SNEDDS NE-12-7 nanoemulsion or its surfactant and cosurfactant. Transport of mannitol in free DPBS solution alone was considered as the control. There was no statistical difference in the total 6-h cumulative transported amount of mannitol between the groups ($p > 0.05$). The results demonstrated that neither the nanoemulsion nor the surfactant and cosurfactant significantly increased the mannitol transport across the cell monolayer. The overall percent mannitol permeated across the monolayer from all the groups were less than 10%.

The transport of propranolol through the MDCK monolayers for the groups studied is show in Fig. 4. The transport of propranolol across MDCK monolayer was significantly decreased ($p < 0.01$) in the presence of SNEDDS NE-12-7 nanoemulsion or its surfactant and cosurfactant, compared with the control (DPBS solution alone).

At the end of the transport study, both apical and basal media were taken and analyzed to calculate the total radioactivity. The results are shown in Table 4. It is very clear that the recovery of radioactivity from the transwells with SNEDDS is comparable to the recovery of the radioactivity from the transwells with DPBS alone, indicating that the presence of SNEDDS did not alter the recovery of radioactivity.

The % relative TEER of MDCK monolayer measured during the transport study shown in Fig. 5 indicates that there was a slight dip in the TEER in the presence of SNEDDS. This decline was not statistically significant ($p > 0.05$).

Table 3
Droplet size of the donor and receiver side during the BLM transport study through MDCK cell monolayer (mean ± S.D., $n = 3$)

Formulation at the donor chamber	Droplet size (nm)				
	Donor chamber	Receiver chamber			
		0 h	2 h	4 h	6 h
FITC-BLM Solution	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
FITC-BLM Solution with SA/CoSA of SNEDDS NE-12-7	12.4 ± 2.0	ND[a]	11 ± 0.7	10.9 ± 0.9	11.3 ± 1.2
FITC-BLM Solution with Blank SNEDDS NE-12-7	38.5 ± 10.6	ND[a]	28.6 ± 2.1	37.6 ± 6.4	36.5 ± 8.2
FITC-BLM-Loaded SNEDDS NE-12-7	32.8 ± 13.4	ND[a]	29.7 ± 3.2	28.5 ± 9.8	31.2 ± 7.9
Blank SNEDDS NE-12-7	31.3 ± 5.4	ND[a]	34.0 ± 4.0	34.6 ± 2.0	31.2 ± 5.7

[a] Not detected.

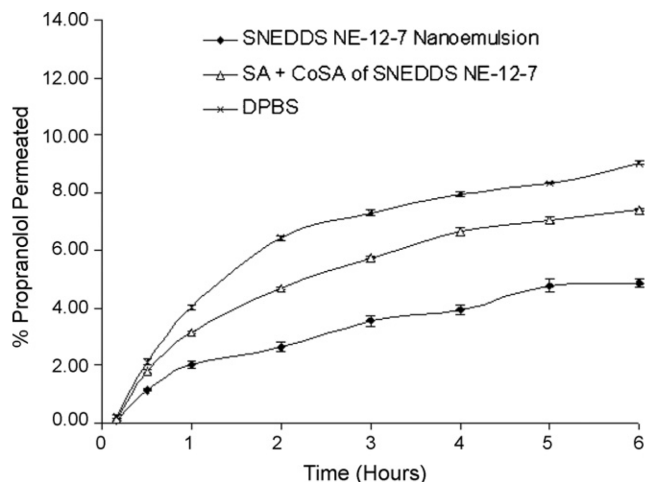

Fig. 4. Effect of SNEDDS NE-12-7 nanoemulsion and its surfactant/cosurfactant on the transport of propranolol through MDCK monolayer. To the donor side of the confluent MDCK monolayer, radio-labeled propranolol was added with or without NE-12-7 nanoemulsion or surfactant/cosurfactant of NE-12-7. Samples were withdrawn from the receiver side at different time points for the measurement of propranolol (mean ± S.D., $n = 3$).

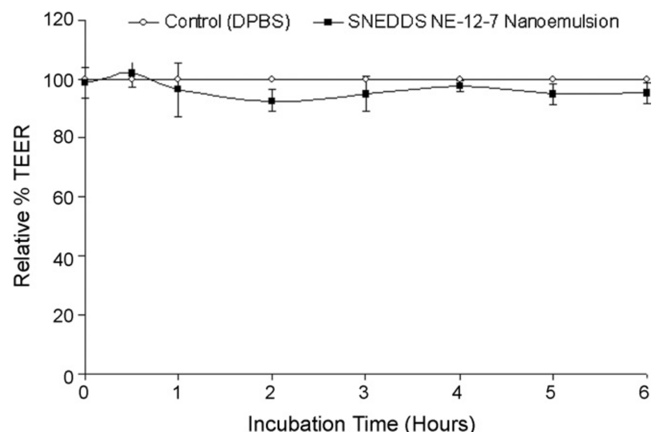

Fig. 5. TEER of the MDCK monolayer in the transwells during the transport study with or without NE-12-7 nanoemulsion (mean ± S.D., $n = 3$).

4. Discussion

The transport study results indicate that BLM in free solution was not able to permeate across the monolayer since it is a large hydrophilic protein. But when BLM was loaded into SNEDDS NE-12-7 and formed nanoemulsion, the transport of BLM significantly increased (almost 25–33 fold) across the monolayer. However, the BLM transport was not much increased when BLM solution mixed with SNEDDS NE-12-7 and then formed nanoemulsion, in which case the BLM was in the aqueous phase. These results show that, only when BLM was dissolved into SNEDDS, the resultant nanoemulsion was able to significantly enhance the transport of BLM. This indicates that the absorption of BLM occurred within the oil droplets. It is well known that microemulsion droplets can be efficiently absorbed intact via simple passive diffusion or pinocytosis or endocytosis (Georgakopoulos et al., 1992). When the oil droplet absorbed through cell monolayer, the BLM which was loaded in the oil droplet was effectively absorbed, but not the BLM which was outside the oil droplets. One of the present results that the oil droplets of the nanoemulsion were transported across the cell monolayer further supported the idea that BLM was transported simultaneously with the oil droplets. Therefore, it is very critical to load the protein drug inside the oil phase of the nanoemulsion in order to enhance the protein drug absorption. These results suggest that SNEDDS may efficiently transport protein drugs across biological membrane *in vivo*, which has been demonstrated in our *in vivo* oral absorption study (Venkata Ramana Rao et al., in press).

In order to confirm that the fluorescent assay in the transport study is the measurement of FITC-BLM but not the free FITC which might be cleaved from the FITC-BLM, FITC solution was used as one of the controls in the transport study. The free FITC solution showed much higher transport rate than the FITC-BLM cases regardless of whether the FITC-BLM is in free solution or in nanoemulsion. These results indicate that FITC-BLM was stable during this transport study since if FITC-BLM had been cleaved to liberate free FITC, there would have been a lot of transport of FITC in the cases other than BLM-loaded SNEDDS, especially in the case of the free solution of FITC-BLM. Therefore, the fluorescence measurement in this study reflects the transport of BLM.

Without the formation of nanoemulsion, the surfactant Cremophor EL and cosurfactant Transcutol HP did increase the permeation of BLM across the monolayer, but only to a very limited extent (3.9 folds), which is far less than the increase by the nanoemulsion (24.9 folds). It is known that macromolecular permeability can be enhanced by the use of surfactants via paracellular transport since the surfactants may alter the epithelial barrier properties (Hochman and Artursson, 1994). These results further verify the fact that only when BLM is loaded into the oil phase, there is an efficient absorption of BLM across the monolayer, since the fine oil droplets can take transcellular route to cross the cell membrane. Hence, protein-loaded SNEDDS may be a much more efficient dosage form for non-invasive protein drug delivery than the preparations which contain just the surfactant and cosurfactant as the absorption enhancer.

SNEDDS NE-12-7 nanoemulsion showed much higher transport of BLM as compared to the other 15 different SNEDDS. This demonstrates that the enhancement of BLM transport by nanoemulsion mainly dependent on the SNEDDS components, meaning the oil, the surfactant and the cosurfactant, and the ratio of these components. In case of SNEDDS NE-12-7 and NE-12-5, they have the same oil, surfactant and cosurfactant but the ratio of these compo-

Table 4
Recovery of mannitol and propranolol from the transwells in the monolayer integrity study

Formulation	% Recovery			
	MDCK monolayer		No monolayer	
	Mannitol	Propranolol	Mannitol	Propranolol
SNEDDS NE-12-7 Nanoemulsion	92.92 ± 5.2	91.48 ± 3.96	98.51 ± 1.78	98.56 ± 2.12
SA and CoSA of SNEDDS NE-12-7	91.73 ± 4.57	93.1 ± 3.56	97.12 ± 2.91	97.41 ± 2.79
DPBS (pH 7.4)	90.36 ± 3.65	99.3 ± 2.56	90.60 ± 2.48	92.69 ± 2.01

Confluent MDCK monolayer (donor side) was exposed to various formulations together with radio-labeled mannitol and propranolol for 6 h. Then the total radio activity at both the donor and receive sides was measured, and expressed as the as percentage of the initial radioactivity (mean ± S.D., $n = 3$).

nents is different and hence the transport of BLM from these two SNEDDS are also different. The former contains higher percentage of the surfactant and cosurfactant, indicating that the more the surfactant/cosurfactant in the formula, the higher the transport rate. SNEDDS prepared with Caproyl-90 (oil) along with Cremophor EL (surfactant) and Transcutol HP (cosurfactant), significantly reduced the transport of BLM as compared to SNEDDS with Lauroglycol FCC as oil along with the same surfactant and cosurfactant. And when oil, Caproyl-90 and surfactant, Cremophor EL were kept at the same ratio of 4:3 and changing the cosurfactant from Transcutol HP to propylene glycol reduced the transport of BLM by 2 fold.

It is noticed that the values of BLM transport rate are different in two different transport studies. For example, SNEDDS NE-12-7 nanoemulsion shows 33% BLM transport in the screening study (Fig. 1) and 22% BLM transport in the second study with all the controls (Fig. 2). This difference may be because these studies were conducted on a different passage number and in entirely different time period. So the monolayer formation may have been a little bit different in the two transport studies. But the trend in the amount of BLM transported across the monolayer in these two studies is very similar.

It is very critical to monitor the integrity of the cell monolayer when it is used for the *in vitro* transport study in order to make sure the monolayer is not broken or disrupted. Therefore, the potential effect of SNEDDS on the MDCK monolayer integrity during the transport studies was examined by both a paracellular and a transcellular marker. Mannitol has a $\log P$ (partition coefficient) value of -2.5 and molecular weight of 182.2. It usually undergoes passive paracellular transport and thus has been used as a paracellular transport marker (Cogburn et al., 1991; Kaushal et al., 2006). Propranolol has a $\log P$ value of 2.53 and molecular weight of 259.3, and undergoes passive paracellular transport. Therefore, it has been used as a transcellular transport marker (Cogburn et al., 1991; Kaushal et al., 2006).

The results show that the transport of mannitol in all cases was very similar and less than 10%. It has been reported that less than 10% mannitol permeation across the monolayer is acceptable in order to confirm that the integrity of the monolayer is maintained (Palamakula and Khan, 2004). Therefore, it can be concluded that neither the nanoemulsion nor the surfactant/cosurfactant significantly compromised the integrity of the monolayer compared to the pure PBS solution. The nanoemulsion and the surfactant/cosurfactant slightly increased the mannitol transport after 3 h, which indicated the possibility of slight opening of the tight junction between the cells. BLM transport data also indicate the same. The opening of tight junction is desired for protein drugs as well as other hydrophilic drug delivery, because these drugs cannot diffuse through the cell membranes and they usually take paracellular route via the junctions between the cells. However, the present results indicate that the opening caused by the surfactant and cosurfactant or the nanoemulsion was very minimal, and significant enhancement of the transport of these hydrophilic molecules could not be achieved.

It is very interesting that the presence of nanoemulsion or surfactant/cosurfactant actually decreased the transport of propranolol. The main reason might be that the presence of SNEDDS components increased the viscosity of the donor chamber which hindered the movement of propranolol. The other possible reason might be some of the nanoemulsion droplets covered the monolayer surface, which became a barrier for propranolol to diffuse through the monolayer.

5. Conclusion

The transport of a hydrophilic protein, BLM across the MDCK monolayer was significantly increased when loaded into the SNEDDS formulation (wherein BLM is dissolved in the oil phase which then forms nanoemulsion spontaneously upon the addition of aqueous media). The SNEDDS system loaded with the protein drugs in its oil phase may be used to effectively increase the absorption of the protein drugs.

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