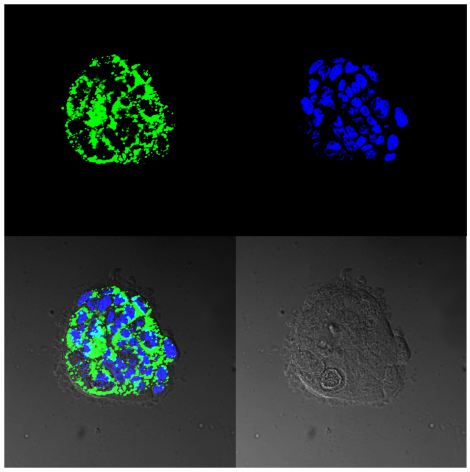
Cell Line Studies



Chapter 6

6.1. Introduction

The ultimate goal in drug research is to develop safe and efficient drugs with favourable pharmacokinetic characteristics suitable for oral route. Since the oral route is considered to be the most convenient route of drug delivery, knowledge of all the factors influencing drug absorption following peroral administration is of major importance. The first physical and biochemical barrier to limit drug absorption is the epithelium of the intestinal mucosa. Intestinal absorption of a drug is, of course, one of the element of interest in bioavailability studies [1] .

Since *in vivo* absorption studies in animals are complex, time-consuming, ethically challenging and expensive, there has been a recognized need to develop alternative *in vitro* methods [2]. Cell culture models are used as intermediately complex systems between whole animal studies and isolated enzymes, membrane fractions or artificial lipid bilayers [3].

Caco-2 is a widely used, well differentiated cell line derived from human colorectal adenocarcinoma. Permeability across Caco-2 cell monolayers is considered to model intestinal absorption, ever since the cells correspond to many of the characteristics and functions of the epithelium of the small intestine [4]. Caco-2 cell line has been well categorized during the decades of its use and is currently approved as the "golden standard" of intestinal cell models [5]. Along with the intestinal-like passive permeability characteristics, numerous active influx and efflux transporters and metabolic enzymes have been observed in fully differentiated Caco-2 cell monolayers [6].

The purpose of present study was to evaluate cell viability, quantitative and qualitative cellular uptake, transport mechanism and permeability of all prepared formulations viz. DE and NISO SMEDDS and NE in the Caco-2 cell system.

6.2. Materials and Methods

6.2.1. Materials

DE and NISO was received as gift samples from Alembic Research Centre, Vadodara, India. Fetal Bovine Serum (FBS), Trypan Blue, Trypsin - EDTA 1X Solution, Minimum Essential Medium Eagle (MEM) and Antibiotic Antimycotic solution 100X liquid (Penicillin-streptomycin solution) were procured from Himedia Lab. Pvt. Ltd. India. 12-well Transwell inserts were purchased from Nunc, Denmark. Well plates of 6, 24 and 96 were purchased from Costar, Corning, USA. Coumarin-6 and MTT dyes were purchased from Sigma Aldrich, Mumbai, India. Caco-2 cell line was purchased from National Centre for Cell Science (NCCS), India. All other chemicals used were of analytical reagent grade.

6.2.2. Methods

Caco-2 cell lines are well known for the over-expression of P-gp efflux transporters and additionally a build up model for *in vitro* oral drug absorption. Caco-2 cell lines were obtained from NCCS, Pune, India and the cell passages between 35 and 40 were used in the experiment. The cells were cultured in 50 cm^2 tissue culture flasks. MEM medium with Earle's salts, 2mM L-Glutamine, 1mM Sodium pyruvate, NEAA and 1.5 g/L sodium bicarbonate, supplemented with 10% Fetal Bovine Serum (Origin: Brazil, EU Approved, Gamma irradiated), and 1% Antibiotic Antimycotic Solution with 10,000 U Penicillin, 10mg Streptomycin and 25 µg Amphotericin B per mL in 0.9% normal saline was used as culture medium. The cell line was incubated at 37°C in humidified atmosphere containing 5% CO_2 in Jouan IGO150 incubator (Thermo-Fisher, Waltham, USA). Media was changed after every 2–3 days and sub-culturing was done when cell confluency became more than 70–80%. Trypsin-EDTA solution containing 0.25% trypsin, 0.038% EDTA in Hanks' Balanced Salt Solution w/o Calcium and Magnesium was used to detach the cells.

6.2.2.1. Preparation of dye loaded formulations

Coumarin-6, a lipophillic fluorescent model dye, was used for the preparation of dye loaded SMEDDS and NE formulations similar to procedure as explained in chapter 4 and 5, (sections 4.3.4.and 5.2.3 respectively) where the drug was replaced with Coumarin-6 dye, for cell uptake studies to allow their visualization by means of fluorescent microscopy [7].

6.2.2.2. Cell viability study

Cell viability (Caco2-cell line) studies were performed to investigate the safety aspects of the surfactants and co-surfactants used to formulate DE and NISO SMEDDS and NE. Cell viability study was carried out by 3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum bromide (MTT) assay on the previously grown Caco-2 cell monolayer. Tissue culture flasks containing Caco-2 cells were trypsinized and the cell suspension was suitably diluted to fixed volume. The cells were then counted using hemocytometer using trypan blue as staining dye. Successively, cells were first cultured in a 96-well plate at a seeding density of 1.0×10^4 Caco-2 cells/well and incubated for 24h. Preparations were diluted with MEM culture medium to different concentrations viz. 100µg/mL, 80µg/mL and 60µg/mL for optimized DE SMEDDS, DE NE, DE suspension, DE SMEDDS placebo and DE NE placebo. In case of NISO, preparations were diluted with MEM culture medium to different concentrations viz. 60µg/mL, 40µg/mL and 20µg/mL for optimized NISO SMEDDS, NSIO NE, NISO suspension, NISO SMEDDS placebo

and NE placebo. After incubation, the cells were treated separately with 150 µL of above sample preparations and incubated at 37°C in CO_2 incubator for 4h. The cells were also treated with Triton-X100 which acted as positive control and phosphate buffer pH 7.4 as negative control. After 4h of incubation, the medium was removed and 150µL of MTT reagent (1 mg/mL) in serum free medium was added to each well. The plates were then incubated at 37°C for another 4h. At the end of the incubation period, the medium was removed and the intracellular formazan was solubilized with 150 µL DMSO and quantified by reading the absorbance at 570 nm with a reference filter of 620 nm using Micro plate multi detection instrument (680-XR, Bio-Rad Laboratories, France). Percentage cell viability was calculated based on the absorbance measured relative to that of cells exposed to the negative control (Phosphate buffer saline) [8, 9].

6.2.2.3. Cell uptake study by FACS

Caco-2 cell line was used to determine cell uptake of lipophilic dye (Coumarin-6) using FACS. For the study, dye loaded formulations were prepared as explained above (6.2.2.1). The cell uptake study was carried out by seeding 1.0×10^6 Caco-2 cells/well in 6 well plates for 48h. For time-dependent uptake, the cells were treated with 100µL each of dye loaded optimized formulations (equivalent to 1 mg/mL) and plain dye solution at predetermined time intervals of 1 and 4h and incubated at 37°C in Jouan IGO150 CO_2 incubator (Thermo-Fisher, Waltham, USA). At the end of time points, the culture medium was washed twice with PBS (pH 7.4). Further, the cells were trypsinized with Trypsin-EDTA solution and centrifuged at 6000 rpm for 1 min to get cell pellet. The cells were then resuspended in FACS buffer (9.8mL PBS+ 0.1mL FBS+100 mg BSA), passed through strainer (0.20µ) and were analysed using FACS (FACS Canto-II,BD Biosciences, San Jose, USA) using BD FACS Diva 6.1.3 software, BD Biosciences, USA [10].

6.2.2.4. Qualitative cell uptake study by Confocal microscopy

The qualitative cell uptake study was carried out by seeding 1.0×10^5 Caco-2 cells on rounded glass cover slips at bottom of 6 well plates for 24 h. On reaching 80% confluency, the culture medium was replaced with HBSS. After 30 min of incubation at 37ºC, cell monolayers were washed three times with HBSS for 5 min. The cells were then incubated with 100 µL of 100 µg/mL (for DE) and 60 µg/mL (for NISO) of Coumarin-6 dye solution and dye loaded formulations. To investigate time dependent uptake, the cells were then incubated for 60 and 120 min. After the specified incubation period, the cells were removed from the medium and washed

twice with PBS (pH 7.4) for visual observation using optical microscope (Nikon Digital Sight DS-Fi2, Japan) for evaluating fluorescence intensity as a function of cellular uptake. The cell monolayers were then fixed with 70% ethanol solution for 20 min and rinsed with HBSS. After rinsing, the nuclei were counter stained with DAPI (4',6-diamidino-2-phenylindole) for 3 min and rinsed again with HBSS, mounted in glycerol and localization of dye loaded formulations were observed using confocal laser scanning microscope (LSM 710, Carl-Zeiss Inc., San Diego, USA). The images were analyzed by Zen imaging software using 430 nm excitation and 485 nm emission wavelength, (Green fluorescence) for Coumarin-6 and 350 nm excitation and 470 nm emission wavelength, (Blue fluorescence) for DAPI [10, 11].

6.2.2.5. Cell permeability study using Transwell Insert

The cell permeability study was carried out by seeding $5x10^3$Caco-2cells/insert in Transwell® inserts-3470-clear (6.5 mm diameter inserts, 0.4 µm pore size, Corning, NY14831) for 21 days. Media was changed every second day for first 7 days followed by every alternate day thereafter. The integrity of the monolayers was checked by monitoring the permeability of the paracellular leakage marker, Lucifer yellow across the monolayers combined with transepithelial electrical resistance (TEER) measurement using EVOM-Epithelial Volt-ohmmeter fitted with planar electrodes (World Precision Instruments, Sarasota, FL). Those monolayers with TEER more than 800 Ω•cm2 were used in the transport studies. The cell monolayers were considered tight enough for the transport experiments when the apparent permeability coefficient (Papp) with Lucifer yellow was less than 0.5×10^{-6} cm/s [5]. All transport studies were conducted at 37°C. The transport buffer containing 150 µL of drug suspension and SMEDDS formulation were added on the apical (0.5 mL) side while the basolateral side of the inserts contained 1.5 mL of the transport buffer. After 30, 60, 120, 180 and 240 min of incubation, 100µL aliquot was withdrawn from the receiver chamber and was immediately replenished with an equal volume of pre-warmed HBSS at 37ºC [5]. The concentration of the test compounds in the transport medium was immediately analyzed by HPLC technique using reported method [5]. The apical-to-basolateral permeability coefficient (P_{app} in cm/s) was calculated according to following equation:

$$P_{app} = \frac{dQ/dt}{A*C_0*60} \qquad \text{....Eqn 6.2.1}$$

where dQ/dt is the amount of respective formulation (DE-SMEDDS, DE NE, NISO SMEDDS and NISO NE) in basolateral compartment as a function of time (mg/min), A is the monolayer

area (cm^2), and C_0 is the initial concentration of drug in apical compartment (mg/mL) [10]. Enhancement ratio (ER) was calculated using following formula:

$$ER = \frac{P_{app} \text{ of formulation}}{P_{app} \text{ of drug suspension}} \quad \text{..... Eqn 6.2.2.}$$

6.3. Results and Discussion

6.3.1 Cell viability study

Safety of all optimized DE and NISO formulations, their respective suspensions and placebo were assessed by MTT assay through Caco-2 cells. Since Caco-2 cell lines were used as *in vitro* absorption barrier, safety/toxicity of formulation on this absorption barrier was checked before performing transport studies. The viability studies on Caco-2 cells were performed for 4 h. Figure 6.3.1 represents the concentration versus percent viability data of cells incubated with all optimized formulations of DE and NISO individually along with Triton-X 100 and Phosphate buffer saline, PBS (negative control). Reduction in the percent viability was observed for cells incubated with Triton-X 100 (Positive control). The percent cell viability data for Caco-2 cells was found to be >80% for all formulations, at all the studied concentrations. The cell viability was higher in case of DE SMEDDS, DE NE, NISO SMEDDS, NISO NE than their respective drug suspensions. These results suggested that the drug when encapsulated inside the oil carriers becomes safer for the intestinal tissues than direct drug suspension. Results also indicated that the surfactant and co surfactant did not cause any cytotoxicity at the levels studied and hence could be termed safe and non toxic. Hence, we can say all the developed formulations are safer than drug suspension.

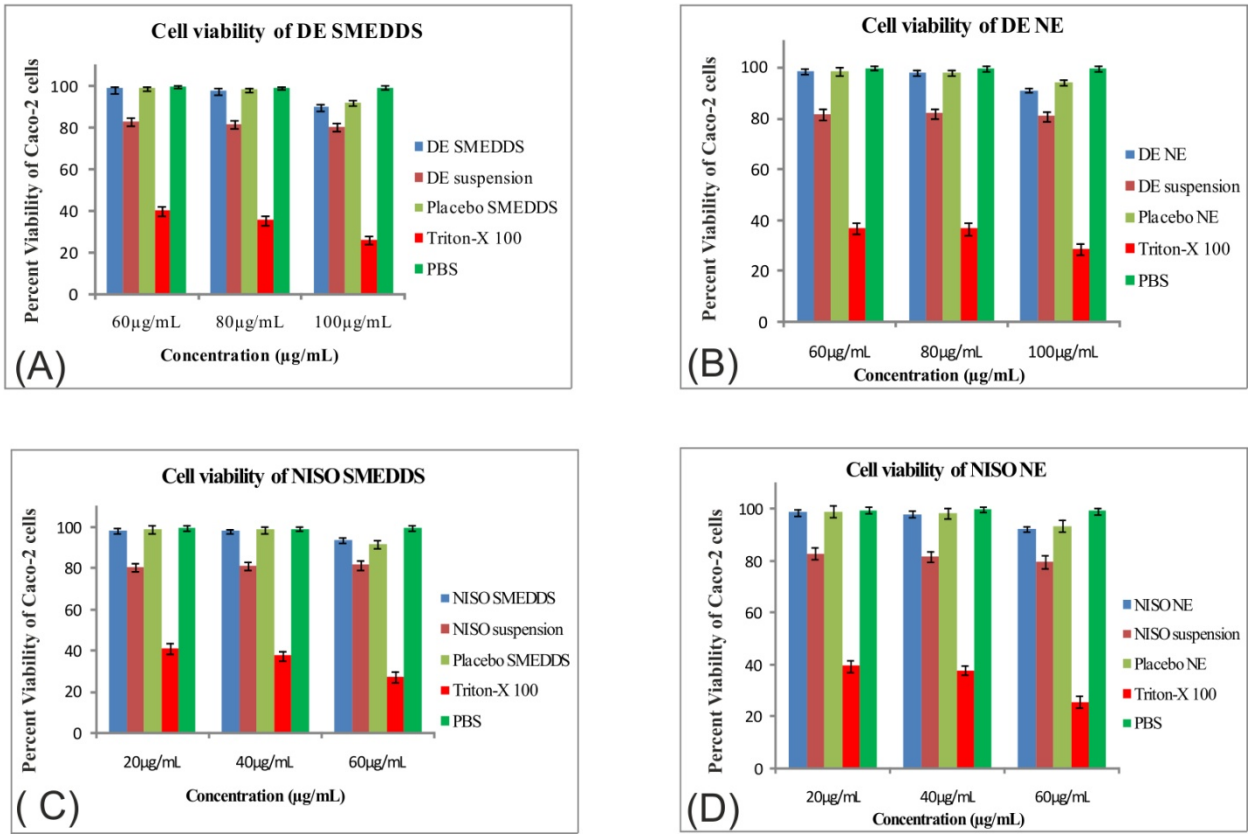


Figure 6.3.1: Bar chart depicting the percent cell viability for (A) DE-SMEDDS, DE suspension, Placebo SMEDDS, Phosphate buffer saline (PBS) and Triton-X 100 (B) DE-NE, DE suspension, Placebo NE, Phosphate buffer saline (PBS) and Triton-X 100 (C) NISO-SMEDDS, NISO suspension, Placebo SMEDDS, Phosphate buffer saline (PBS) and Triton-X 100 (D) NISO-NE, NISO suspension, Placebo NE, Phosphate buffer saline (PBS) and Triton-X 100

6.3.2. Cell uptake study by FACS

Relative extent of uptake of 6-coumarin-loaded SMEDDS and NE for both the drugs in comparison to their respective plain dye solutions were analyzed by FACS in Caco-2 cells. Caco-2 cells are reported to over express P-gp and cytochrome P450 enzymes [12]. FACS uptake studies showed that the fluorescence intensity inside the cells increased with SMEDDS and NE formulations when compared with plain dye solutions. The fluorescent intensity clearly showed a significantly higher uptake and internalization for DE SMEDDS and DE NE (Figure 6.3.2), NISO SMEDDS and NISO NE (Figure 6.3.3) than their respective plain dye solutions. The enhancement in fluorescence by prepared SMEDDS and NE were almost more than doubled from 1 to 4 h, which supported the superiority of developed formulations over plain drug

solution in cell uptake. This was confirmed through mean fluorescent intensity (MFI) which shows the relative extent of intracellular uptake of formulations. MFI with Coumarin-6 loaded SMEDDS and NE formulations showed almost similar trend of increased intensity as compared to plain dye solution as depicted in Figure 6.3.4 and Figure 6.3.5. MFI with Coumarin-6 loaded SMEDDS was found to be 1.78 and 1.92 times higher after 1 h and 5.30 and 9.92 times higher after 4 h than plain dye solutions for DE and NISO respectively. MFI with Coumarin-6 loaded NE was found to be 4.39 and 4.64 times higher after 1 h and 7.03 and 13.04 times higher after 4 h than plain dye solution for DE and NISO respectively. Thus, enhanced uptake of SMEDDS and NE by Caco-2 cells implies greater intestinal absorption and can be correlated with enhanced therapeutic activity of both the drugs [10].

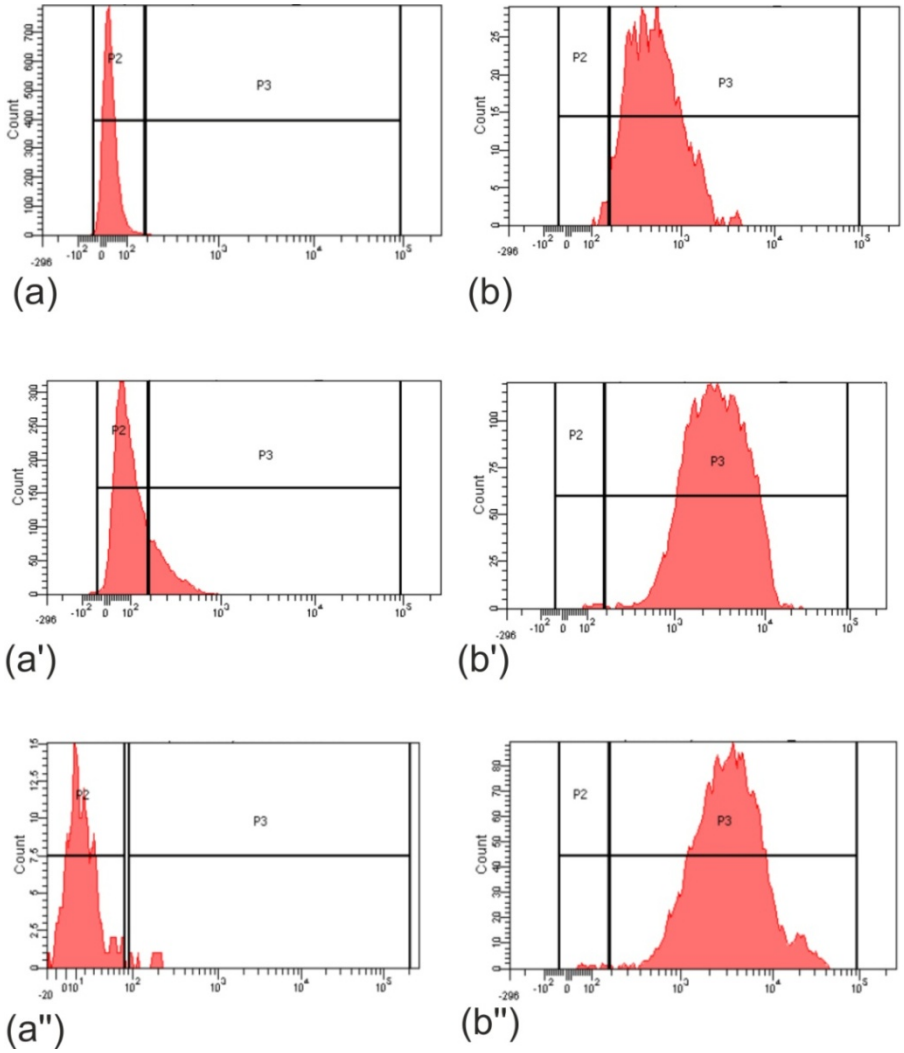


Figure 6.3.2. Cell uptake studies by FACS for coumarin-6 loaded dye solution (a, b); coumarin-6 loaded SMEDDS (a', b') and coumarin-6 loaded NE (a'', b'') at 1 h (a, a', a'') and 4 h (b, b', b'') in Caco-2 cells for DE

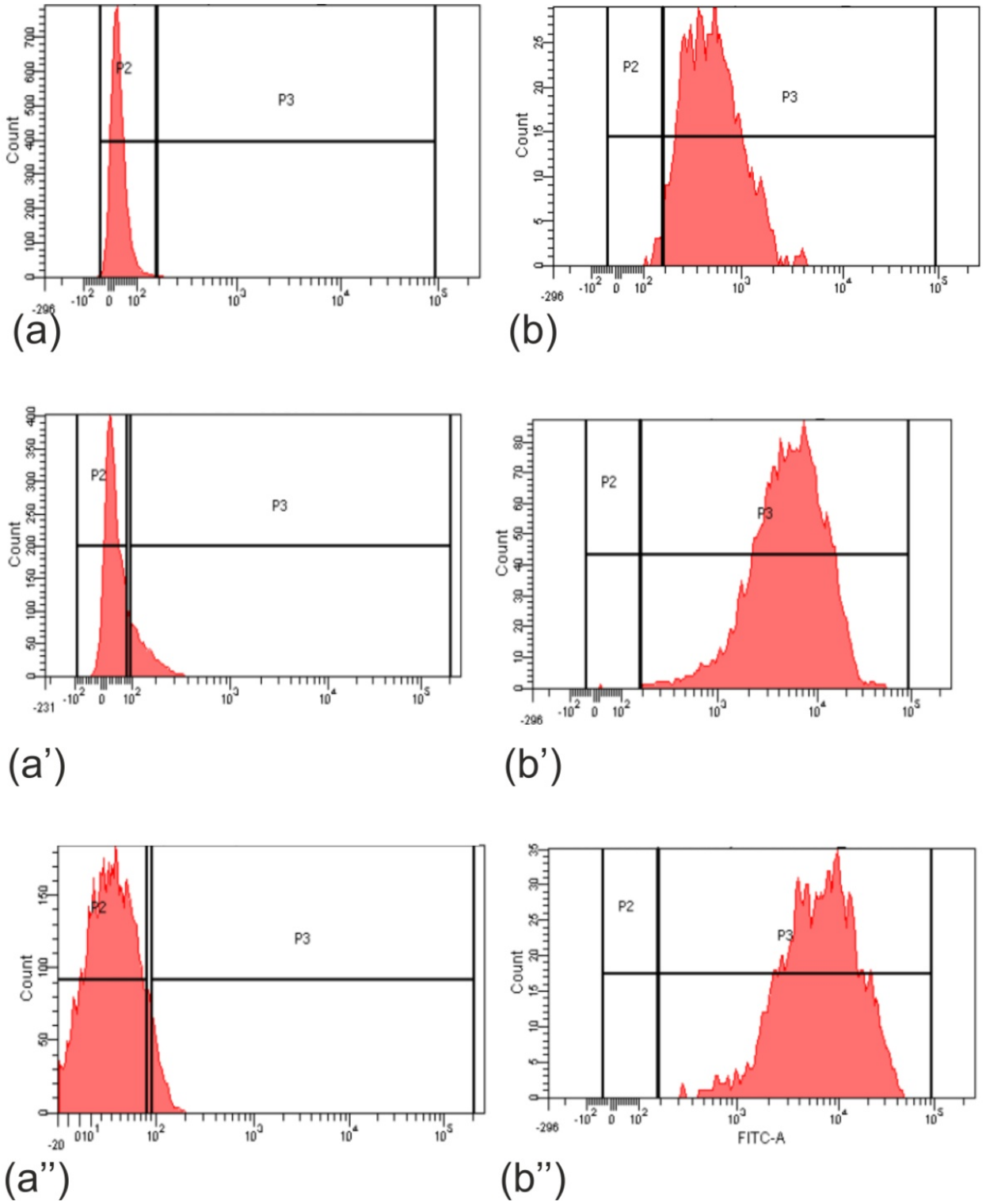


Figure 6.3.3. Cell uptake studies by FACS for coumarin-6 loaded dye solution (a, b); coumarin -6 loaded SMEDDS (a', b') and coumarin -6 loaded NE (a'', b'') at 1 h (a, a', a'') and 4 h (b, b', b'') in Caco-2 cells for NISO

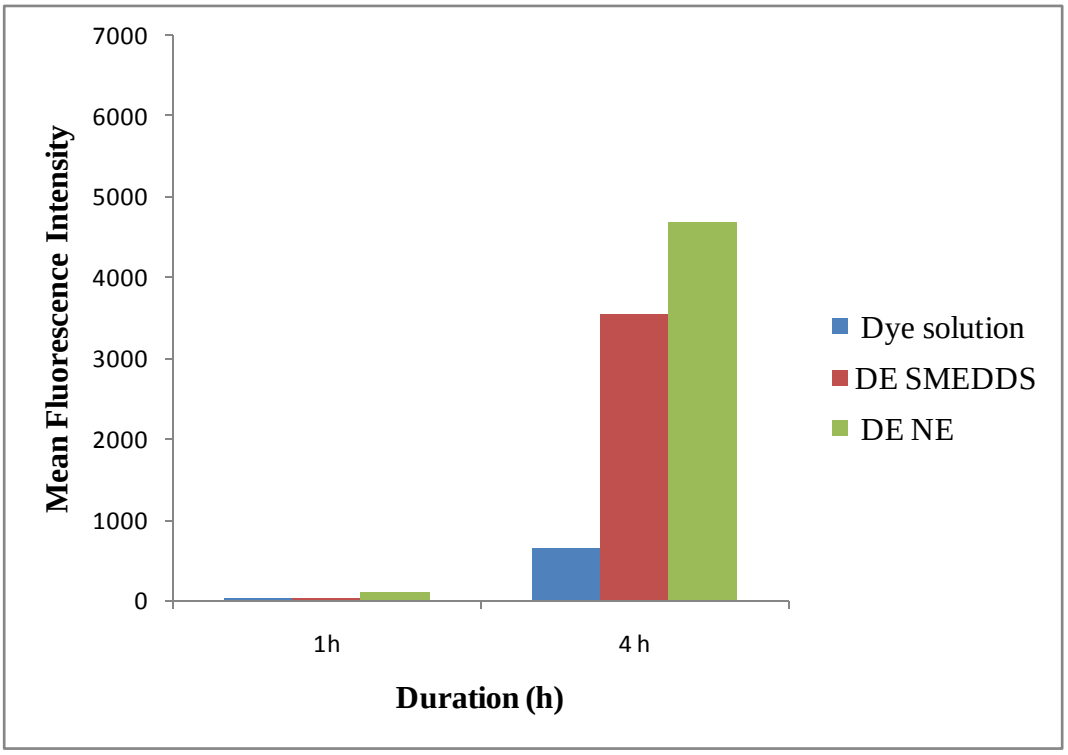


Figure 6.3.4: Mean fluorescence intensity plot of dye solution, Coumarin-6 loaded SMEDDS and Coumarin-6 loaded NE for DE

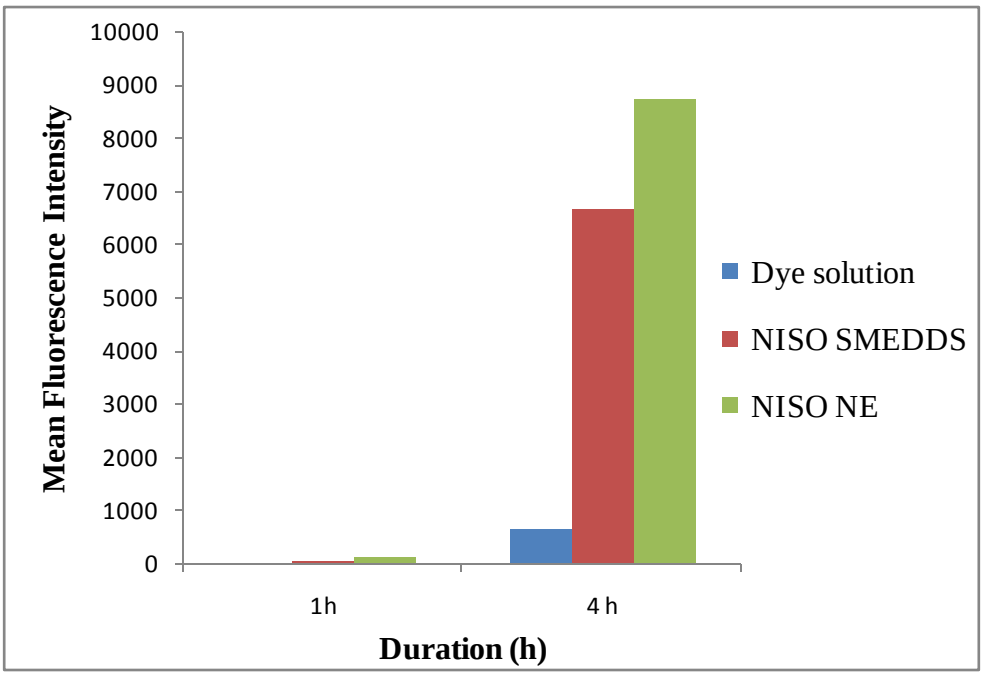


Figure 6.3.5: Mean fluorescence intensity plot of dye solution, Coumarin-6 loaded SMEDDS and Coumarin-6 loaded NE for NISO

6.3.3. Qualitative cell uptake study by Confocal microscopy

Cellular uptake and distribution of coumarin-6 loaded SMEDDS, coumarin-6 loaded NE and coumarin-6 solution was examined by confocal microscopy using Caco-2 cells. Coumarin-6 was chosen as hydrophobic model dye to mimic hydrophobic nature of drug. The uptake of SMEDDS and NE by Caco-2 cells was time dependent and increased with incubation time. The confocal micrograph images showed enhanced fluorescent intensity inside cells for SMEDDS and NE of both the drugs as depicted in Figures 6.3.6 to 6.3.10, when compared to coumarin-6 solution (Figure 6.3.6) after 1 h and 4 h incubation which implies the enhanced absorption through the M cells of Peyer's patches of intestine. This enhanced permeation by prepared formulations can be attributed to the presence of surfactants viz. Cremophor EL and Trasncutol HP, as potential absorption enhancers which may amend epithelial barrier property [13]. Small particles are more efficiently taken up due to greater hydrophobic interactions with the membrane of Caco-2 cells [14]. Therefore, improved permeation across cell membrane might be due to small particle sizes of nanoformulations and presence of excipients [15]. Hence, it could be clear from the results that intracellular uptake of hydrophobic drug like DE and NISO could be enhanced by encapsulating them in the lipid based nanoformulations and can lead to enhanced oral bioavailability.

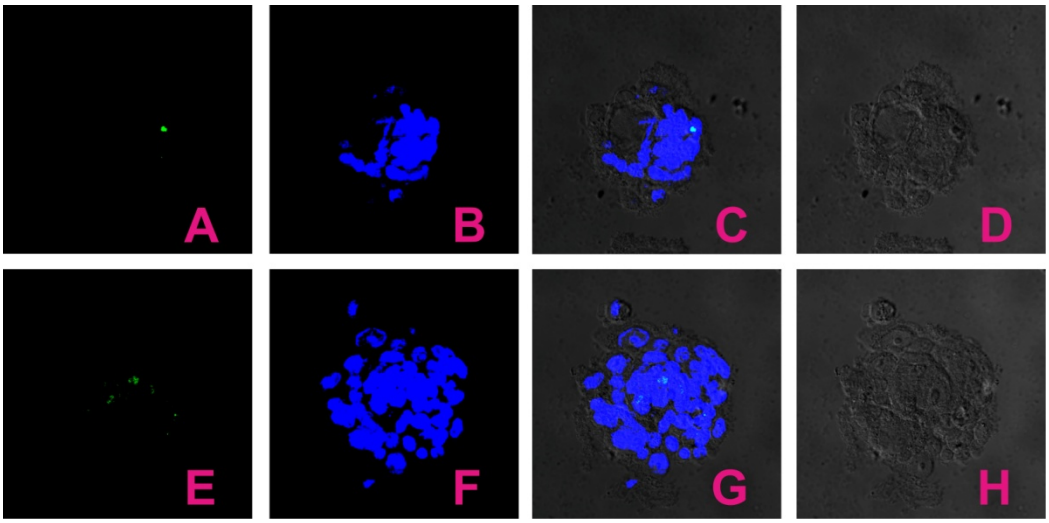


Figure 6.3.6. Qualitative cellular uptake of Coumarin-6 solution in Caco-2 cells; at 1 h (A–D) and 4 h (E-H); Green flourescence spots represents dye solution, Blue fluorescence represents DAPI-stained nuclei, (C and G) represents overlapped images and (D and H) represents Differential Interference Contrast (DIC) images showing cells

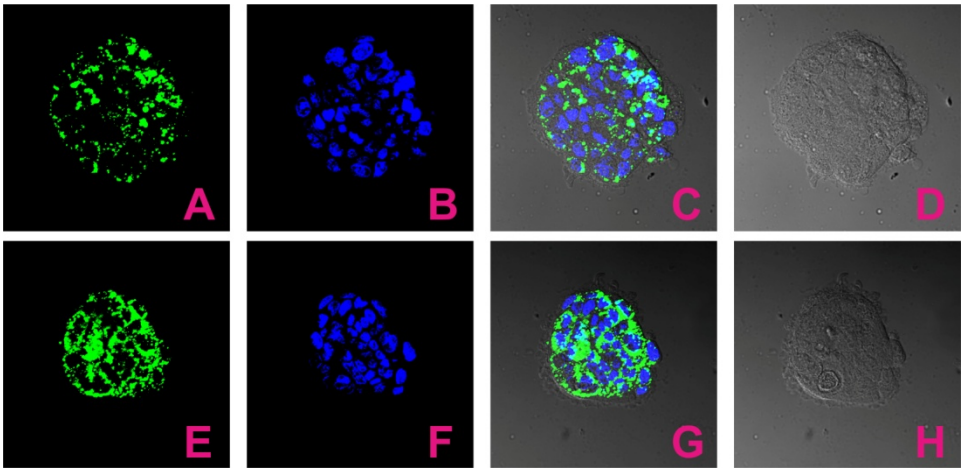


Figure 6.3.7. Qualitative cellular uptake of DE SMEDDS in Caco-2 cells; at 1 h (A–D) and 4 h (E-H); Green flourescence spots represents dye-loaded SMEDDS, Blue fluorescence represents DAPI-stained nuclei, (C and G) represents overlapped images and (D and H) represents Differential Interference Contrast (DIC) images showing cells

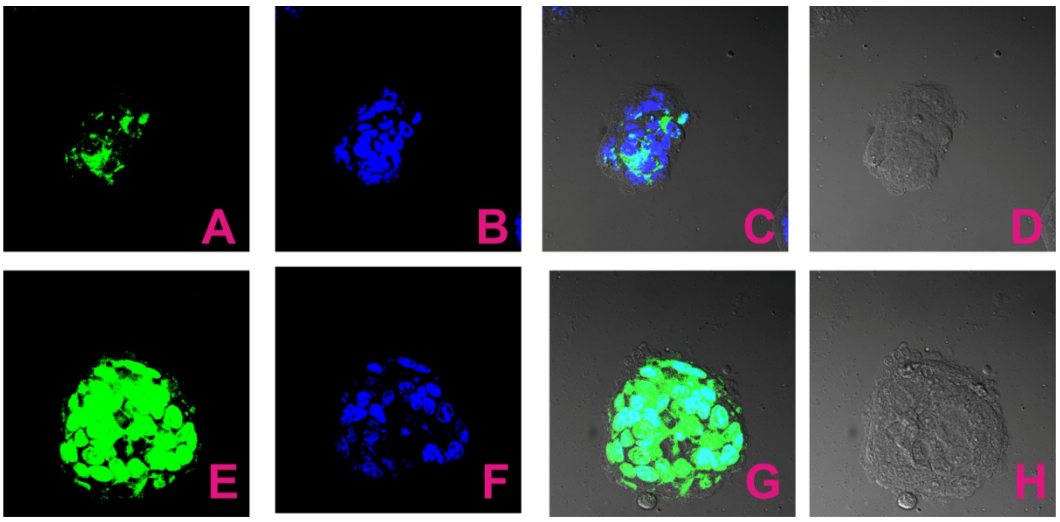


Figure 6.3.8. Qualitative cellular uptake of DE NE in Caco-2 cells; at 1 h (A–D) and 4 h (E-H); Green flourescence spots represents dye-loaded NE, Blue fluorescence represents DAPI-stained nuclei, (C and G) represents overlapped images and (D and H) represents Differential Interference Contrast (DIC) images showing cells

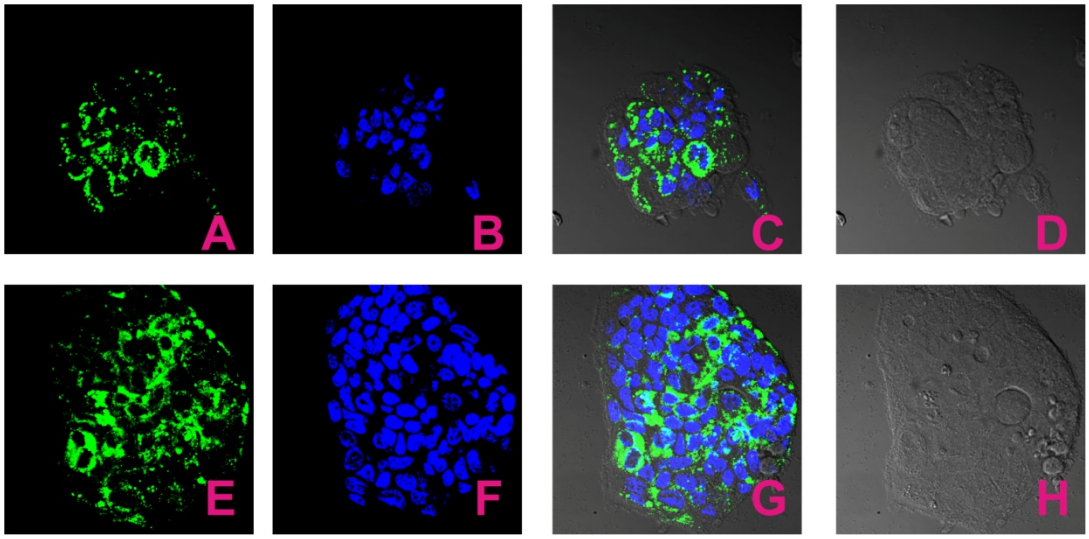


Figure 6.3.9. Qualitative cellular uptake of NISO SMEDDS in Caco-2 cells; at 1 h (A–D) and 4 h (E-H); Green flourescence spots represents dye-loaded SMEDDS, Blue fluorescence represents DAPI-stained nuclei, (C and G) represents overlapped images and (D and H) represents Differential Interference Contrast (DIC) images showing cells

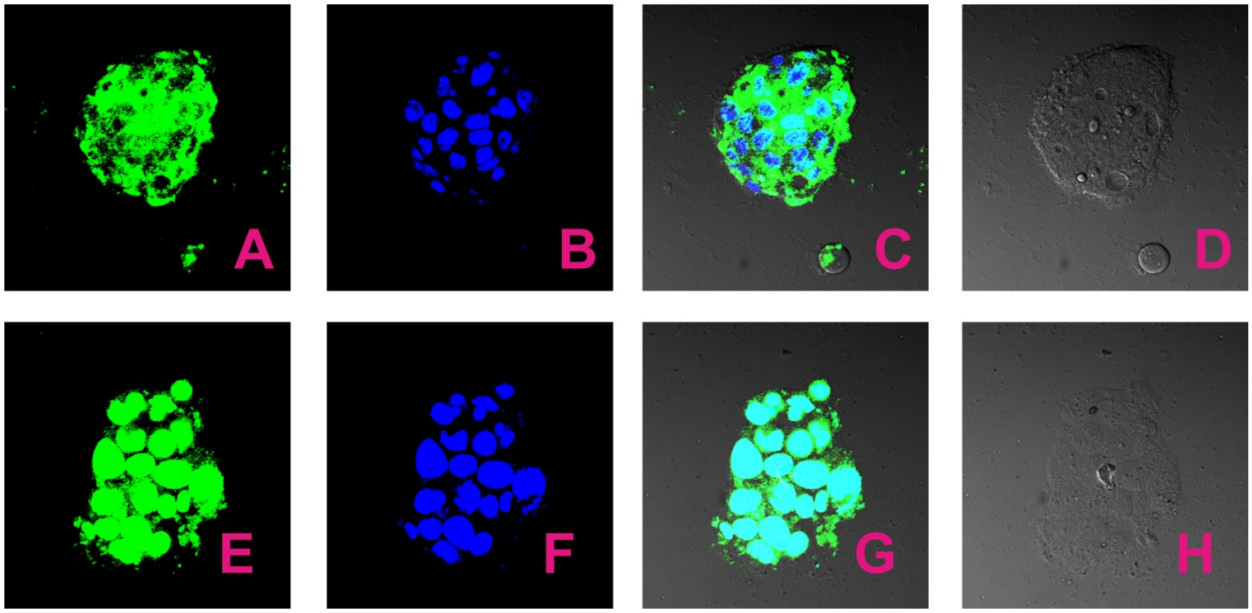


Figure 6.3.10. Qualitative cellular uptake of NISO NE in Caco-2 cells; at 1 h (A–D) and 4 h (E-H); Green flourescence spots represents dye-loaded NE, Blue fluorescence represents DAPI-stained nuclei, (C and G) represents overlapped images and (D and H) represents Differential Interference Contrast (DIC) images showing cells

6.3.4. Cell permeability study using Transwell Insert

The Caco-2 cell model has been the most extensively characterized and useful cell model in the field of drug permeability study. As the permeation characteristics of drugs across Caco-2 cell monolayers correlates with that of human intestinal mucosa, it has been suggested that Caco-2 cells can be used to predict the oral absorption of drugs in humans. Transepithelial permeability of DE SMEDDS and NE was measured at concentration of 100µg/mL, while that for NISO SMEDDS and NE was measured at 60µg/mL as negligible toxicity towards Caco-2 cells was found at these concentrations during MTT assay of the same. The average P_{app} for Lucifer yellow with Caco-2 cells was found to be 0.41±0.11 x 10^{-6} cm/sec, 0.38±0.08 x 10^{-6} cm/sec, 0.39±0.12 x 10^{-6} cm/sec, 0.35±0.10 x 10^{-6} cm/sec 0.37±0.03 x 10^{-6} cm/sec and 0.40±0.09 x 10^{-6} cm/sec for DE suspension, DE SMEDDS, DE NE, NISO suspension, NISO SMEDDS and NISO NE respectively which confirmed the integrity and suitability of monolayers for further experiment. Also the TEER value for Caco-2 cells grown on filters after 21 days was found to be 913 Ωcm^2, 898 Ωcm^2 , 905 $\Omega cm^{2,}$, 936 Ωcm^2, 911 Ωcm^2 and 942 Ωcm^2 for DE suspension, DE SMEDDS, DE NE, NISO suspension, NISO SMEDDS and NISO NE respectively indicating the presence of tight junctions and good integrity of the monolayer. The amount of drug transfered from DE formulations and DE suspension across Caco-2 monolayer were as shown in table 6.3.1 and that of NISO are as shown in table 6.3.2.

Table 6.3.1: Drug transfered across the Caco-2 cell line for DE SMEDDS, DE NE and DE suspension

Time (min)	Amount of drug transfered (mg)		
	DE SMEDDS	DE NE	DE Suspension
30	0.011	0.015	0.003
60	0.030	0.047	0.006
120	0.065	0.087	0.014
180	0.133	0.117	0.020
240	0.147	0.149	0.024
	dQ/dt= 0.0005	dQ/dt= 0.0007	dQ/dt= 0.0001

Table 6.3.2: Drug transfered across the Caco-2 cell line for NISO SMEDDS, NISO NE and NISO suspension

Time (min)	Amount of drug transfered (mg)		
	NISO SMEDDS	NISO NE	NISO Suspension
30	0.013	0.023	0.003
60	0.032	0.058	0.006
120	0.065	0.093	0.015
180	0.119	0.128	0.021
240	0.148	0.150	0.0025
	dQ/dt= 0.0006	dQ/dt= 0.0008	dQ/dt= 0.0001

The apparent permeability and enhancement ratio of all prepared formulations and their respective drug suspensions were calculated as per eqn 6.2.1 and 6.2.2 respectively. Reults are as shown in Table 6.3.3 and Table 6.3.4.

Table 6.3.3: Apparent permeability (P_{app}) and enhancement ratio (ER) of DE Suspension, DE SMEDDS, DE NE.

	DE Suspension	DE SMEDDS	DE NE
Papp (cm/s)	0.966×10^{-6}	4.779×10^{-6}	5.863×10^{-6}
ER	--	4.95	6.07

Table 6.3.4: Apparent permeability (P_{app}) and enhancement ratio (ER) of NISO Suspension, NISO SMEDDS and NISO NE

	NISO Suspension	NISO SMEDDS	NISO NE
Papp (cm/s)	0.992×10^{-6}	5.023×10^{-6}	6.908×10^{-6}
ER	--	5.02	6.91

Results showed that there was increase in permeability of the developed SMEDDS and NE formulations as compared to respective drug suspensions.This was attributed to the higher uptake of SMEDDS and NE by endocytosis in Caco-2 cells. (If the P_{app} value of a compound is less than 1×10^{-6} cm/sec, in between $1\text{-}10 \times 10^{-5}$ cm/ sec, and more than 10×10^{-6} cm/sec, it can be classified as poorly (0-20%), moderately (20-70%) and well (70-100%) absorbed compounds,

respectively) [16, 17]. Plain drug suspensions were poorly absorbed while the lipid based nanoformulations showed marked increase in their absorption. This might be because of small particle size of SMEDDS and NE formulations, combined with amphiphilic nature of non ionic surfactants present in the formulations. Additionally, Capmul MCM C8 along with Cremophor EL inhibits P-gp-function and Cremophor EL also shows CYP3A inhibitory activity, which will further enhance the intestinal absorption of various drugs [15, 18].

REFERENCES

1. S. Deferme, P. Annaert, and P. Augustijns, In Vitro Screening Models to Assess Intestinal Drug Absorption and Metabolism, in *Drug Absorption Studies: In Situ, In Vitro and In Silico Models*, C. Ehrhardt and K.-J. Kim, Editors. 2008, Springer US: Boston, MA. p. 182-215.
2. EMEA. Committee for proprietary medicinal products (CPMP): Replacement of animal studies with in vitro models. 1997 [cited 2017 24.12.2017]; Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003354.pdf.
3. A. Quaroni and J. Hochman, Development of intestinal cell culture models for drug transport and metabolism studies. Advanced drug delivery reviews, 1996. 22(1-2): p. 3-52.
4. A.R. Hilgers, R.A. Conradi, and P.S. Burton, Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharmaceutical research, 1990. 7(9): p. 902-910.
5. I. Hubatsch, E.G. Ragnarsson, and P. Artursson, Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nature protocols, 2007. 2(9): p. 2111-2119.
6. D. Sun, H. Lennernas, L.S. Welage, J.L. Barnett, C.P. Landowski, D. Foster, D. Fleisher, K.-D. Lee, and G.L. Amidon, Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. Pharmaceutical research, 2002. 19(10): p. 1400-1416.
7. I. Rivolta, A. Panariti, B. Lettiero, S. Sesana, P. Gasco, M. Gasco, M. Masserini, and G. Miserocchi, Cellular uptake of coumarin-6 as a model drug loaded in solid lipid nanoparticles. Journal of Physiology and Pharmacology, 2011. 62(1): p. 45.
8. G. Joshi, A. Kumar, and K. Sawant, Enhanced bioavailability and intestinal uptake of Gemcitabine HCl loaded PLGA nanoparticles after oral delivery. European Journal of Pharmaceutical Sciences, 2014. 60: p. 80-89.
9. S. Beg, G. Sharma, K. Thanki, S. Jain, O. Katare, and B. Singh, Positively charged self-nanoemulsifying oily formulations of olmesartan medoxomil: systematic development, in vitro, ex vivo and in vivo evaluation. International journal of pharmaceutics, 2015. 493(1): p. 466-482.
10. G. Joshi, A. Kumar, and K. Sawant, Bioavailability enhancement, Caco-2 cells uptake and intestinal transport of orally administered lopinavir-loaded PLGA nanoparticles. Drug delivery, 2016. 23(9): p. 3492-3504.
11. L.M. Negi, M. Tariq, and S. Talegaonkar, Nano scale self-emulsifying oil based carrier system for improved oral bioavailability of camptothecin derivative by P-Glycoprotein modulation. Colloids and Surfaces B: Biointerfaces, 2013. 111: p. 346-353.
12. C. Awortwe, P. Fasinu, and B. Rosenkranz, Application of Caco-2 cell line in herb-drug interaction studies: current approaches and challenges. Journal of pharmacy & pharmaceutical sciences: a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques, 2014. 17(1): p. 1.
13. X. Sha, G. Yan, Y. Wu, J. Li, and X. Fang, Effect of self-microemulsifying drug delivery systems containing Labrasol on tight junctions in Caco-2 cells. European Journal of Pharmaceutical Sciences, 2005. 24(5): p. 477-486.
14. M. Liang, I.-C. Lin, M.R. Whittaker, R.F. Minchin, M.J. Monteiro, and I. Toth, Cellular uptake of densely packed polymer coatings on gold nanoparticles. ACS nano, 2009. 4(1): p. 403-413.

15. B.D. Rege, J.P. Kao, and J.E. Polli, Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers. European journal of pharmaceutical sciences, 2002. 16(4): p. 237-246.
16. S. Yee, In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man—fact or myth. Pharmaceutical research, 1997. 14(6): p. 763-766.
17. P. Artursson and J. Karlsson, Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochemical and biophysical research communications, 1991. 175(3): p. 880-885.
18. Microemulsions for drug solubilization and delivery, in *Drug delivery strategies for poorly water-soluble drugs*, D. Douroumis and A. Fahr, Editors. 2012, John Wiley & Sons.