Chapter 7
In-vitro & Ex-vivo Studies

Dermal Delivery of Protein/Peptide Based Antimicrobial to Treat Secondary Infection in Psoriasis and Eczema

7.1 Introduction

The optimized formulations of Omiganan and DPK 060 were subjected to various *in-vitro* and *ex-vivo* studies i.e., *in-vitro* drug release, *in-vitro* cytotoxicity, cellular uptake, *in-vitro* antimicrobial efficacy, *ex-vivo* drug permeation, and skin retention studies.

7.2 Materials And Instruments

List of Materials and Reagents

Chemical/Reagents	**Manufacturer / Supplier**
Omiganan	S-Biochem, Kerala (Custom synthesis)
DPK-060	S-Biochem, Kerala (Custom synthesis)
Methanol (A.R. & HPLC Grade)	Spectrochem Pvt. Ltd., Mumbai
Dimethyl sulfoxide	Himedia Pvt. Ltd, Mumbai
Dialysis membrane (12-14 KDa cut-off)	Himedia Pvt. Ltd, Mumbai
Sodium chloride (AR)	S.D. Fine Chemicals, Mumbai
Sodium hydroxide (AR)	Spectrochem Labs Ltd, Vadodara
Potassium chloride (AR)	Spectrochem Labs Ltd, Vadodara
Potassium dihydrogen phosphate (AR)	Spectrochem Labs Ltd, Vadodara
Sodium dihydrogen phosphate (AR)	Spectrochem Labs Ltd, Vadodara
Hydrochloric acid (AR)	Rankem chemical, Vadodara
3T3-fibroblast cells	National Centre for Cell Sciences, Pune
6 and 96-well cell culture plates	Sigma Aldrich, USA
Dulbecco's Modified Eagle Medium (DMEM – high glucose)	Sigma Aldrich, USA
Fluorescein-5-isothiocyanate (FITC)	Sigma Aldrich, USA
Fetal bovine serum (FBS)	Sigma Aldrich, USA
Trypsin-EDTA solution (1X)	Sigma Aldrich, USA
Antibiotic antimycotic solution (100X)	Sigma Aldrich, USA
Thiazolyl blue tetrazolium bromide (MTT)	Sigma Aldrich, USA
Distilled water	Prepared In-house

List of Equipments

Equipments	Manufacturer / Supplier
Digital Weighing Balance	Shimadzu, Japan
RP-HPLC with UV Detector (gradient)	Agilent OpenLab CDS EZChrom, India
Magnetic stirrer	Remi equipments Pvt. Ltd, India
CO_2 incubator	Jouan, Thermo Fisher Scientific, USA
Microtiter plate reader	Biorad, India
Microtome	LEICA RM 2125 RTS, India
Confocal microscope	LSM 710, Carl-Zeiss Inc., USA
Fluorescence microscope	Nikon, India
Vortex mixer	Spinix, Japan
Incubator thermo shaker	Bombay Lab. Services, Mumbai
Centrifuge	Remi Instrument, India

7.3 Methods

7.3.1 *In-vitro* drug release study

The *in-vitro* drug release study was done to assess the release profile of drug(s) from the developed formulations. The *in-vitro* release behavior of the active ingredient from the formulation may help to correlate it with the *in-vivo* release of the drug from the formulation targeted to a specific organ. *In-vitro* release profile of Omiganan and DPK 060 from lotion, liposomes, and NLCs was performed using a dialysis bag technique [1]. Briefly, pre-activated dialysis membrane (Mole. Weight – 12-14 KDa cut-off) was mounted between donor and receptor chamber of the diffusion cell. The receptor chamber was filled with release medium (30 ml of PBS pH 7.4: methanol in 8:2 ratio) and kept for equilibrium for half an hour under mild stirring (100 rpm) on a magnetic stirrer. Subsequently, the optimized batches of free Omiganan gel, Omiganan lotion, Omiganan liposomal gel, Omiganan NLC gel, free DPK 060 gel, DPK 060 lotion, and DPK 060 NLC gel (equivalent to 2 mg Omiganan/DPK 060) were placed in a dialysis bag and both ends were sealed. Samples (0.5 ml) were withdrawn at regular

time intervals of 0.5,1,2,3,4,5,6 and 12 h from the receptor chamber, and the same volume (0.5 ml) was replaced by a fresh release media. Analysis of the samples were done by using the developed HPLC method. The drug release kinetics was then assessed by fitting the data in different mathematical models and comparing R^2 values (n=3) [2].

7.3.2 *In-vitro* cytotoxicity and cellular uptake studies

In-vitro cell lines studies are essential as they provide a preliminary evaluation of direct effects on the tissues and cells to set up clinical relevance in pathological states, screening, and understanding of toxicity mechanisms. The objective of the *in-vitro* cytotoxicity study was to evaluate the toxic potential of the prepared formulations of Omiganan and DPK 060, if any, on the 3T3-fibroblast cells. Additionally, the cellular uptake examination was also carried out to assess the intracellular uptake of the developed formulations in 3T3-fibroblast cell line using confocal microscopy.

7.3.2.1 Cell culture handling and sub-culturing protocol

3T3-fibroblast cells were procured from NCCS, Pune, India. The flask was kept in an anaerobic incubator at 37 °C with 5 % CO_2 for 24 h. Later, the culture media was discarded, and the adherent cells were washed with PBS pH 7.4. Added the freshly prepared Trypsin EDTA solution and kept for incubatation at 37 °C for 10 min to detach the adherent cells. Then freshly prepared growth medium was added to stop trypsin activity, and the cell suspension was centrifuged for 5 min at 1500 rpm. Subsequently, the supernatant was removed and cells were further resuspended in a fresh growth media and were transferred into new flasks at a plating density of $1x10^4$ cells/cm^2 and incubated at 37 °C with 5 % CO_2 to facilitate cell growth. The growth media was changed every 3rd day, and passaging was done once the culture attained 90 % confluency [3, 4].

7.3.2.2 MTT assay

MTT assay was performed in the 3T3-fibroblast cells to assess the cytotoxicity of the developed formulations of Omiganan and DPK 060 [5, 6]. Briefly, the cells were seeded with 5×10^3 cells/well cell density in a 96 well microtiter plate using DMEM

supplemented (high glucose) with 10% FBS. The cell culture was grown for 24 h in a CO_2 incubator maintained at 5% concentration and humidified with saturated Copper sulfate solution. After 24 h, cells were washed 3 times using PBS pH 7.4, and 20 µl MTT dye (5mg/mL) solution was added to each well plate. The MTT dye was permitted to react for 4 h under incubator conditions; after that, the cell media in each well plate was replaced with 100 µl of DMSO, and the microtiter plate was shaken gently to dissolve the crystals of formazan. The color of the formazan was evaluated by using a Biorad microtiter plate reader at 570 nm. The absorbance values of cells treated with PBS pH 7.4 were taken as 100% cell viability, and all other treatments were expressed relative to it [7, 8]. Omiganan and DPK 060 (free drug solution and formulations) in 0.01 – 100 µg/ml concentration range were evaluated for the MTT assay. In this study, Triton X 100 and PBS pH 7.4 were used as positive and negative control, respectively.

7.3.2.3 Cellular uptake studies

The *in-vitro* cellular uptake studies of developed formulations were executed on 3T3-fibroblast cells. Briefly, cells were seeded (cell density of 1×10^5 cells/well) in a 6-well plate using DMEM accompanied with 10% FBS. The cell culture was grown for 24 h in a CO_2 incubator maintained at 5% concentration and humidified with a saturated solution of copper sulfate. To evaluate the cellular uptake, FITC tagged Omiganan/DPK 060 was loaded in the formulations. Subsequently, cells were seeded at a cell density of 1×10^5 cells/well in 6-well plates to allow to adhere for 24 h; subsequently, treatment with FITC tagged Omiganan/DPK060 formulations. The cells were washed 3 times with PBS pH 7.4 and were fixed by using 4% w/v paraformaldehyde (1mL/well) solution. The paraformaldehyde was instantly removed after exposure time, and cells were washed thrice with PBS pH 7.4 accompanied by intermittent shaking for each wash to eliminate the traces of paraformaldehyde. Subsequently, the cells were stained with DAPI (nuclei staining) at 1 µg/mL concentration with enough volume to cover the cells and kept for 10 min for dye permeation under protection by aluminum foil. After that, cells were washed once with PBS pH 7.4 and mounted on the glass slide using PBS: glycerin solution (40:60) with

coverslips. The cellular uptake was examined at 1 and 4 h treatment by confocal microscopy [2, 5].

7.3.3 *In-vitro* determination of minimum inhibitory concentration (MIC)

In-vitro antimicrobial study was executed to determine the MIC of the developed formulations of Omiganan and DPK 060 in microbial strains of *S. aureus* and *C. albicans*. Broth micro-dilution technique was used to determine the MIC of developed formulations [9, 10]. Briefly, *S. aureus* and *C. albicans* were seeded in 96-well plates having cell density of 5×10^3 CFU/well (1×10^5 CFU mL^{-1}, 50 µL). Different concentrations (from 100 to 0.125 µg mL^{-1}, 50 µL) of developed formulations in Mueller Hinton Broth media were added to each well. In this study, Vancomycin and Fluconazole were used as +ve control for *S. aureus* and *C. albicans*, respectively. While microbes without any treatment were considered as -ve control. The microbes were incubated under standard conditions up to 24 h and the optical density was measured using a microplate reader at 600 nm (n=3).

7.3.4 *In-vitro* hemocompatibility studies

The study was conducted to assess the compatibility of developed Omiganan formulations with red blood cells (RBCs). Briefly, blood samples were collected from mice by retro-orbital plexus in 2 ml eppendorf tubes. Blood samples were heparinized with 100 µl Heparin solution (500 IU/ml) in 1 ml blood and subjected for centrifugation at 3000 rpm for 8 min at 4°C to separate the RBCs. The resultant RBC pellet (2 % dispersion) was resuspended and washed thrice using normal saline. Subsequently, 1 ml RBC dispersion was added to 1 ml PBS pH 7.4 (-ve control), 1 ml Triton x-100 (1% v/v, +ve control), and 1 ml Omiganan and DPK 060 (1% w/v) loaded formulations. The treated dispersions were incubated for 1h at 37°C. Later, the samples were evaluated under a light microscope for hemocompatibility [11, 12].

7.3.5 Protease sensitivity assay

Bacteria generally produce proteases and peptidases to inactivate the AMPs. Thus, the peptide integrity against proteolysis must be assessed early for native and nano-formulations of peptides. In this study, trypsin was used as a model enzyme. The

proteolytic concentration of trypsin for Omiganan and DPK-060 was examined by trying different ratios of trypsin/peptide/PBS pH 7.4. Lotion and nano-formulations of Omiganan and DPK 060 were incubated at 37ºC with an appropriate trypsin ratio for 4 h. [13]. Negative controls were performed by incubating Omiganan and DPK-060 in PBS pH 7.4 without trypsin in the same incubation conditions. Samples were taken at time intervals of 15 min, 30 min, 45 min, 1 h, 2 h, and 4 h. The concentration/amount of Omiganan/DPK-060 remaining was quantified by the developed gradient HPLC method.

7.3.6 *Ex-vivo* permeation and skin retention studies

Ex-vivo permeation was performed according to the approved animal protocol (MSU/IAEC/2018-19/1832) by the IAEC committee, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara. Briefly, skin samples (mice) were set up in a franz diffusion cell having the 9 mm diameter. Free Omiganan gel, Omiganan lotion, Omiganan liposomal gel, Omiganan NLC gel, free DPK 060 gel, DPK 060 lotion, and DPK 060 NLC gel (equivalent to 2 mg Omiganan/DPK 060) equivalent to 2 mg Omiganan/DPK 060 was added to the donor compartment. The assay conditions were similar as in the *in vitro* release studies. After 12 h, the skin samples were detached from the diffusion cell, and the skin surface was carefully washed by using PBS pH 7.4. Subsequently, the skin samples were cut into small pieces, and were homogenized by using 5.0 mL ethanol. The concentration of Omiganan/DPK-060 deposited into the skin was examined by means of extraction followed by centrifugation process (5000 rpm for 10 min at 25°C) and the Omiganan/DPK 060 concentration was quantified by the developed HPLC method [14, 15].

7.3.7 *Ex-vivo* fluorescence microscopic study

The skin permeation behavior of Omiganan and DPK-060 loaded formulations were further evaluated by fluorescence microscopy. FITC tagged formulations, i.e., free Omiganan gel, Omiganan lotion, Omiganan liposomal and NLC gel, free DPK 060 gel, DPK 060 lotion, and DPK 060 NLC gel, were placed on the skin samples (mice)

as described in the earlier section. The whole experiment was carried out in the dark. After 6 h, skin sectioning was done using cryo-microtome, sections were fixed on the glass slide and observed under the confocal microscope [15].

7.4 Results and Discussion

7.4.1 *In-vitro* drug release study

The % cumulative drug release of Omiganan and DPK 060 from free drug gel and developed formulations at each time point was calculated and shown in tables 7.1 and 7.2, as illustrated in Fig. 7.1 7.2, respectively.

Table 7.1 *In-vitro* drug release study of Omiganan loaded formulations

Time (hr)	% Cumulative release			
	Free Omiganan gel	**Omiganan lotion**	**Omiganan liposomal gel**	**Omiganan NLC gel**
0.5	58.06 ± 2.25	53.63 ± 3.17	20.14 ± 1.12	17.94 ± 1.25
1	91.71 ± 3.01	89.73 ± 2.82	28.72 ± 1.29	26.7 ± 1.63
2	98.20 ± 1.77	97.53 ± 1.95	39.18 ± 1.61	37.85 ± 1.47
3	98.16 ± 1.26	98.15 ± 1.49	48.78 ± 2.07	45.44 ± 1.86
4	98.61 ± 1.45	98.49 ± 1.55	57.17 ± 1.87	54.67 ± 2.14
5	98.18 ± 1.68	98.17 ± 1.38	68.12 ± 2.12	64.86 ± 1.97
6	98.37 ± 1.52	98.52 ± 1.42	78.04 ± 2.26	76.66 ± 2.49
12	--	--	94.97 ± 1.98	93.48 ± 1.75

Table 7.2 *In-vitro* drug release study of DPK 060 loaded formulations

Time (hr)	% Cumulative drug release		
	Free DPK 060 gel	**DPK 060 lotion**	**DPK 060 NLC gel**
0.5	56.77 ± 2.14	51.98 ± 2.98	15.46 ± 1.20
1	90.49 ± 3.20	87.64 ± 2.61	24.56 ± 1.37
2	97.85 ± 1.89	97.26 ± 1.72	33.04 ± 1.72
3	98.30 ± 1.26	98.07 ± 1.43	41.51 ± 1.63

4	98.22 ± 1.38	98.19 ± 1.27	52.20 ± 1.89
5	98.41 ± 1.15	97.99 ± 1.33	62.54 ± 2.07
6	98.35 ± 1.23	98.14 ± 1.11	72.10 ± 1.83
12	--	--	91.55 ± 1.57

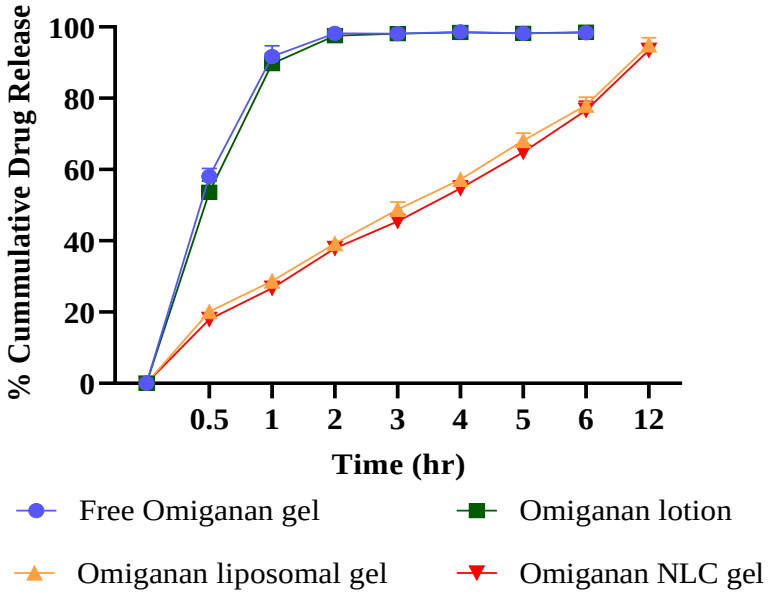


Figure 7.1 *In-vitro* drug release profiles of Omiganan loaded formulations

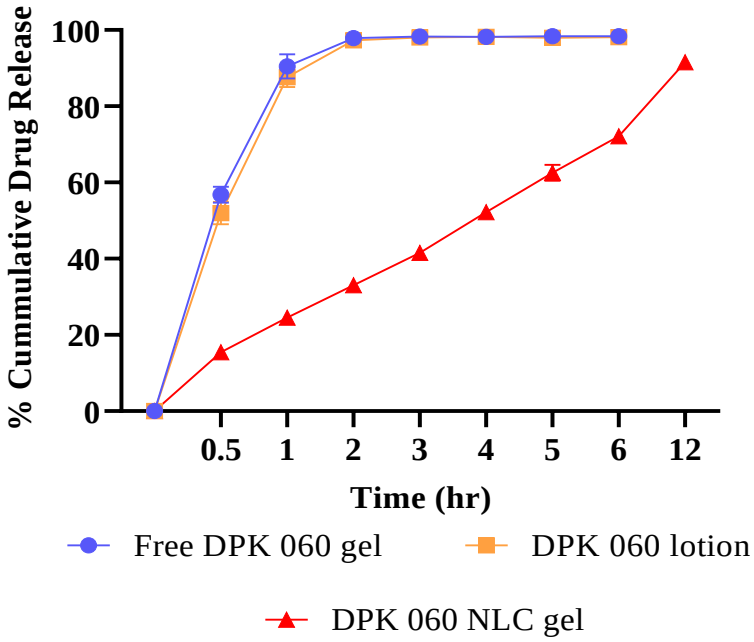


Figure 7.2 *In-vitro* drug release profiles of DPK 060 loaded formulations

Results of i*n vitro* drug release studies demonstrated that around 90% Omiganan was released from the lotion (89.73 ± 2.82 %) and free Omiganan gel (91.71 ± 3.01 %) within 1h. Whereas about 77% of Omiganan was released from the liposomal gel (78.04 ± 2.26 %) and NLC gel (76.66 ± 2.49 %) after 6 h, offers the sustained release of Omiganan. The quick equilibrium was observed from lotion and free Omiganan gel due to the water solubility of the Omiganan.

The result of different mathematical models, i.e., Zero order, First order, Higuchi model, Hixon Crowell, and Korsmeyer- Peppas model, applied to understand the Omiganan release kinetics from liposomal and NLC gel, are presented in Table 7.3.

Table 7.3 Various mathematical models and their correlation coefficient (r^2) values

Mathematical models	**Omiganan liposomal gel (r^2)**	**Omiganan NLC gel (r^2)**
Zero-order	0.896	0.900
First-order	0.990	0.989
Hixon Crowell	0.896	0.901
Higuchi	0.966	0.978
Korsmeyer- Peppas	0.991 (n = 0.59)	0.991 (n = 0.67)

The regression coefficient of the plot of Korsmeyer- Peppas model for Omiganan liposomal gel and NLC gel, was found to be 0.991, which is higher than others. Additionally, the n (release exponent) value was found to be 0.59 and 0.67 for Omiganan liposomal and NLC gel, respectively, i.e., $0.45<n<0.89$ suggests the non-fickian/anomalous diffusion i.e., combination of both diffusion and erosion-controlled release [16, 17].

While the results of *in-vitro* release studies of DPK 060 loaded formulations demonstrated around 84% of DPK 060 was released from the lotion (87.64 ± 2.61) and free DPK 060 gel (90.49 ± 3.20) within 1h. In contrast, around 72% of DPK 060 was released from NLCs after 6 hr, offers the sustained release of DPK 060. The quick equilibrium was observed from lotion and free DPK 060 gel due to the amphiphilic nature of DPK 060.

The result of different mathematical models, i.e., Zero-order, First-order, Higuchi model, Hixon Crowell, and Korsmeyer- Peppas model applied to understand the DPK 060 release kinetics from NLC gel, are presented in Table 7.4.

Table 7.4 Various mathematical models and their correlation coefficient (r^2) values

Mathematical models	**DPK 060 NLC gel (r^2)**
Zero-order	0.914
First-order	0.989
Hixon Crowell	0.914
Higuchi	0.978
Korsmeyer- Peppas	0.993 (n = 0.48)

The regression coefficient of the plot of Korsmeyer- Peppas model for DPK 060 NLC gel, was found to be 0.993, which is higher than others. Additionally, the value of n was found to be 0.48, i.e., $0.45<n<0.89$ suggests the non-fickian/anomalous diffusion i.e., combination of both diffusion and erosion-controlled release [17, 18].

7.4.2 *In-vitro* cytotoxicity and cellular uptake studies

7.4.2.1 MTT assay

The cell viability data for Omiganan and DPK 060 loaded formulations are summarized in Table 7.5 and illustrated in Fig. 7.3 and 7.4, respectively.

Table 7.5 *In vitro* cell viability data for Omiganan and DPK 060 formulations in 3T3-fibroblast cells

Concentration (µg/mL)	% Cell viability						
	Omiganan				DPK 060		
	Solution	Lotion	NLC	Liposomes	Solution	Lotion	NLC
0.01	98.78 ± 2.96	98.23 ± 2.74	98.87 ± 2.40	99.12 ± 2.21	98.16 ± 2.77	98.56 ± 2.39	98.40 ± 1.98
0.1	95.44 ± 3.12	94.35 ± 2.93	97.95 ± 2.95	97.86 ± 2.62	94.67 ± 2.98	95.22 ± 2.73	97.61 ± 2.25
1	90.69 ± 2.87	89.80 ± 3.07	95.72 ± 3.17	94.47 ± 2.99	91.08 ± 3.25	90.51 ± 3.31	94.48 ± 2.60
10	88.49 ± 3.48	87.74 ± 3.20	92.14 ± 3.34	91.93 ± 3.31	89.68 ± 2.59	87.16 ± 2.96	92.94 ± 3.05
50	86.82 ± 4.12	85.97 ± 3.48	91.09 ± 2.92	89.82 ± 2.88	87.60 ± 3.11	84.93 ± 3.43	91.19 ± 2.90
100	80.20 ± 4.41	81.96 ± 3.92	90.21 ± 3.47	88.14 ± 3.95	82.23 ± 3.62	82.88 ± 3.79	90.97 ± 2.58

Note: Cells treated with PBS pH 7.4 were taken as 100% cell viability. % Cell viability of positive control (Triton X 100) was found to be 23.49 ± 3.87 %.

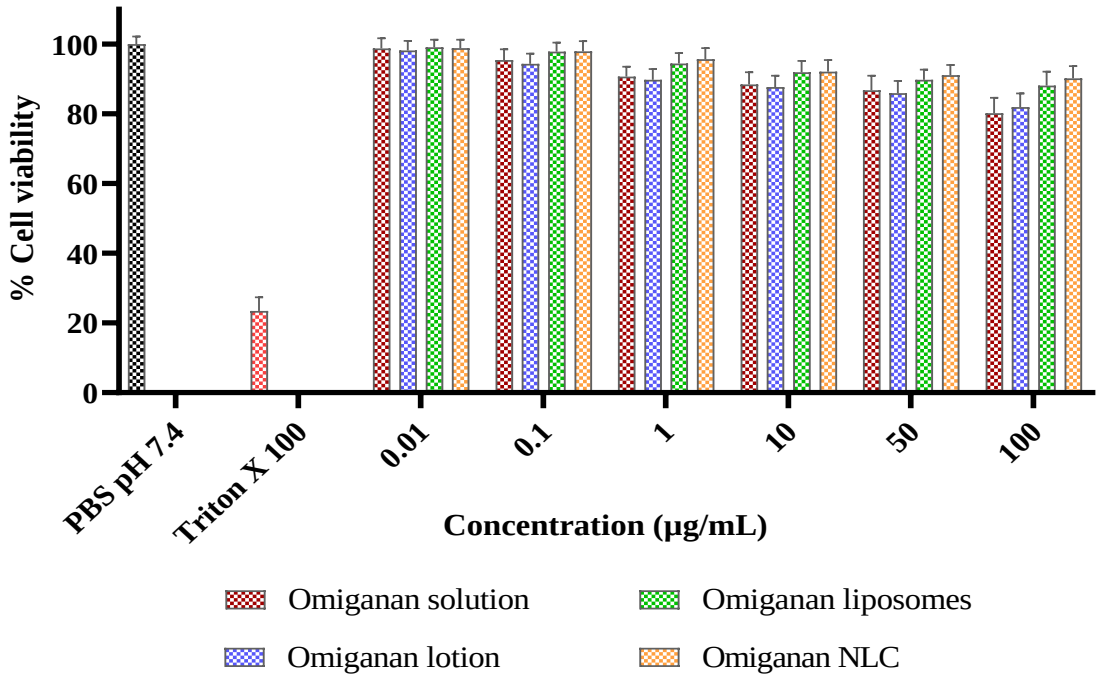


Figure 7.3 *In-vitro* cell viability data for Omiganan formulations in 3T3-fibroblast cells

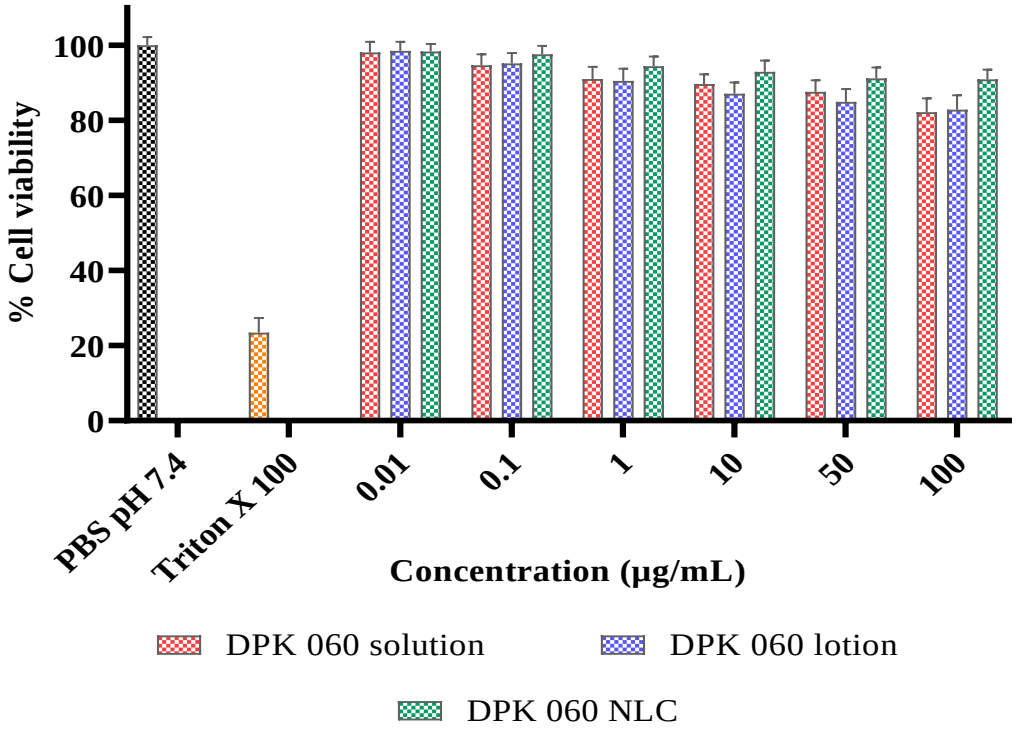


Figure 7.4 *In vitro* cell viability data for DPK-060 formulations in 3T3-fibroblast cells

The viability of cells treated with free Omiganan solution, Omiganan lotion, and Omiganan loaded liposomes and NLCs were found in this order; free Omiganan < Omiganan lotion < Omiganan liposomes < Omiganan NLCs, significantly higher than positive control Triton X100 (<25%). While the viability of cells treated with free DPK 060 solution, DPK 060 lotion, and DPK 060 loaded NLCs were found in this order free DPK 060 < DPK 060 lotion < DPK 060 loaded NLCs, significantly higher than positive control Triton X100 (<25%). The developed formulations of Omiganan and DPK 060 have no potential toxic effects on the 3T3-fibroblast cells and are safe for dermal delivery.

7.4.2.2. Cellular uptake studies

The cellular uptake studies of Omiganan and DPK 060 loaded formulations are demonstrated in Fig. 7.5 and 7.6, respectively. Results showed the higher cellular uptake of the Omiganan loaded liposomal and NLC gel compared to free Omiganan gel and Omiganan lotion after 4 h. In contrast, the results of cellular uptake studies of the DPK 060 formulations demonstrated higher cellular uptake of DPK 060 NLC gel than free DPK 060 gel and DPK 060 lotion after 4 h. The increase in the cellular uptake indicates the enhanced internalization of Omiganan and DPK 060. This may be correlated that the enhanced cellular uptake increases the therapeutic efficacy of Omiganan and DPK 060.

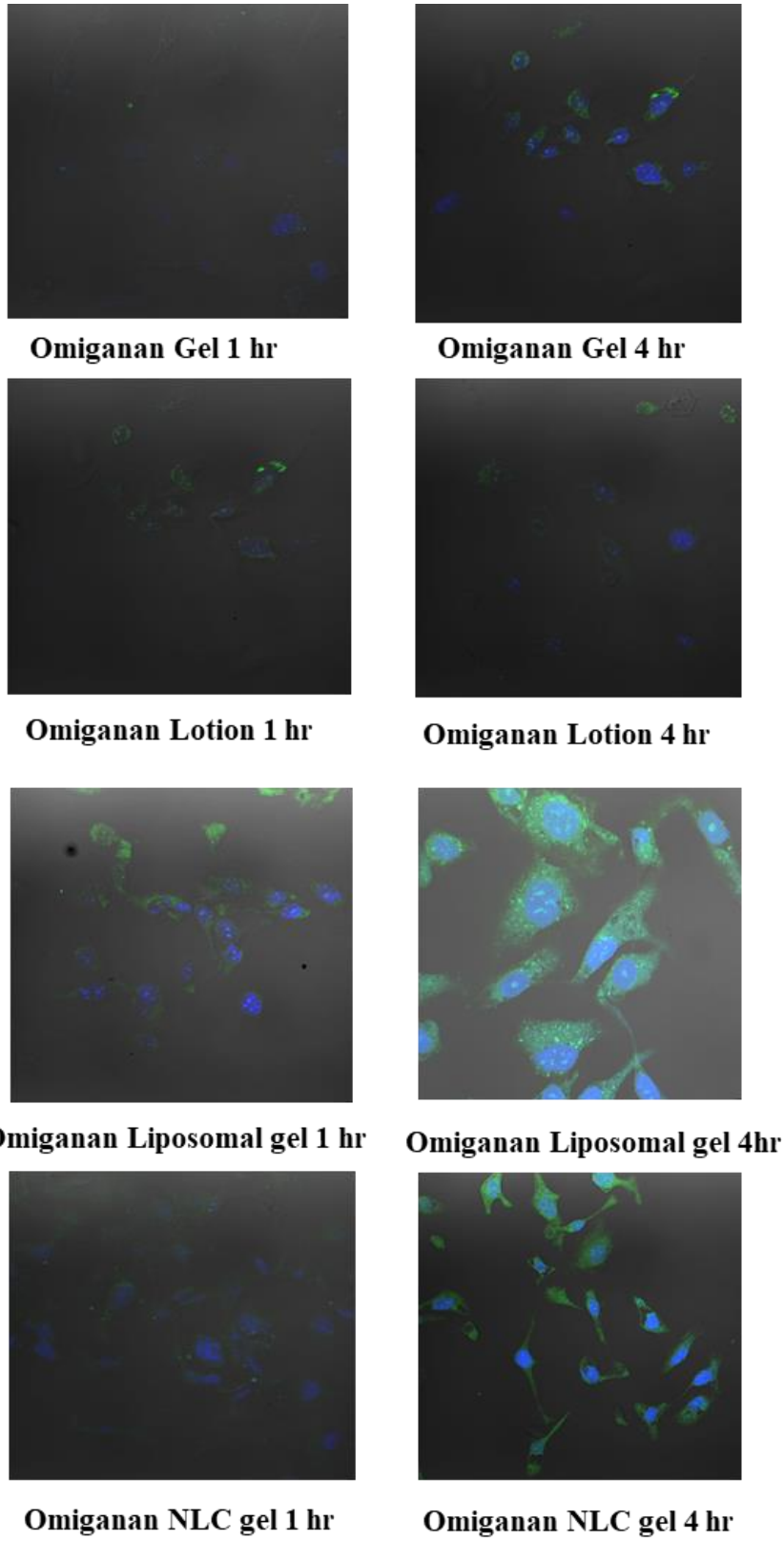


Figure 7.5 *In vitro* cell uptake of Omiganan formulations in 3T3-fibroblast cells

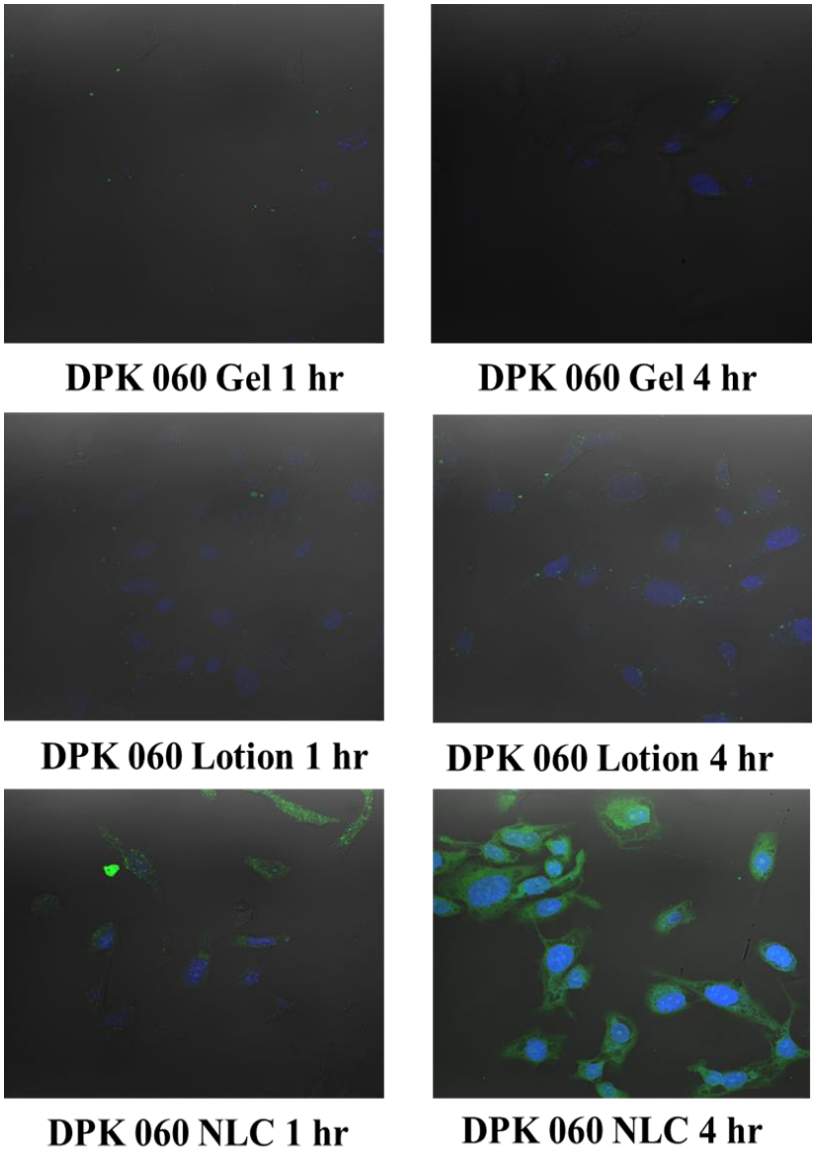


Figure 7.6 *In vitro* cell uptake of DPK 060 formulations in 3T3-fibroblast cells

7.4.3 *In-vitro* determination of MIC

The MIC values for Omiganan and DPK 060 loaded formulations are summarized in Table 7.6. Omiganan loaded formulations exhibited a MIC in the range of 12-16 µg/mL and 32-64 µg/mL against *S. aureus* and *C. albicans*, respectively, in agreement with previously reported MICs [19]. In comparison, DPK 060 loaded formulations exhibited a MIC in the range of 2-4 µg/mL and 128-160 µg/mL against *S. aureus* and *C. albicans*, respectively, in agreement to previously reported MICs [20, 21]. The results indicate that

both of these peptides have strong antibacterial activity against *S. aureus* while less potent to the strain of *C. albicans*.

Table 7.6 MIC values of different formulations of Omiganan and DPK 060 against *S. aureus* and *C. albicans*

Formulations	MIC (µg/mL)	
	S. aureus	***C. albicans***
Vancomycin (positive control)	2	--
Fluconazole (positive control)	--	1
Omiganan solution	12	32
Omiganan lotion	16	64
Omiganan liposomes	16	64
Omiganan NLC	16	64
DPK 060 solution	2	128
DPK 060 lotion	4	160
DPK 060 NLC	4	160

7.4.4 In-vitro hemocompatibility studies

The results of the hemocompatibility study of Omiganan and DPK 060 loaded formulations are shown in Fig. 7.7. The results demonstrated the compatibility of the Omiganan and DPK 060 loaded formulations with the RBCs in comparision to the +ve control; Triton x-100 (hemolytic agent) shows the rupture/lysis of RBCs. The developed formulations were safe and hemocompatible.

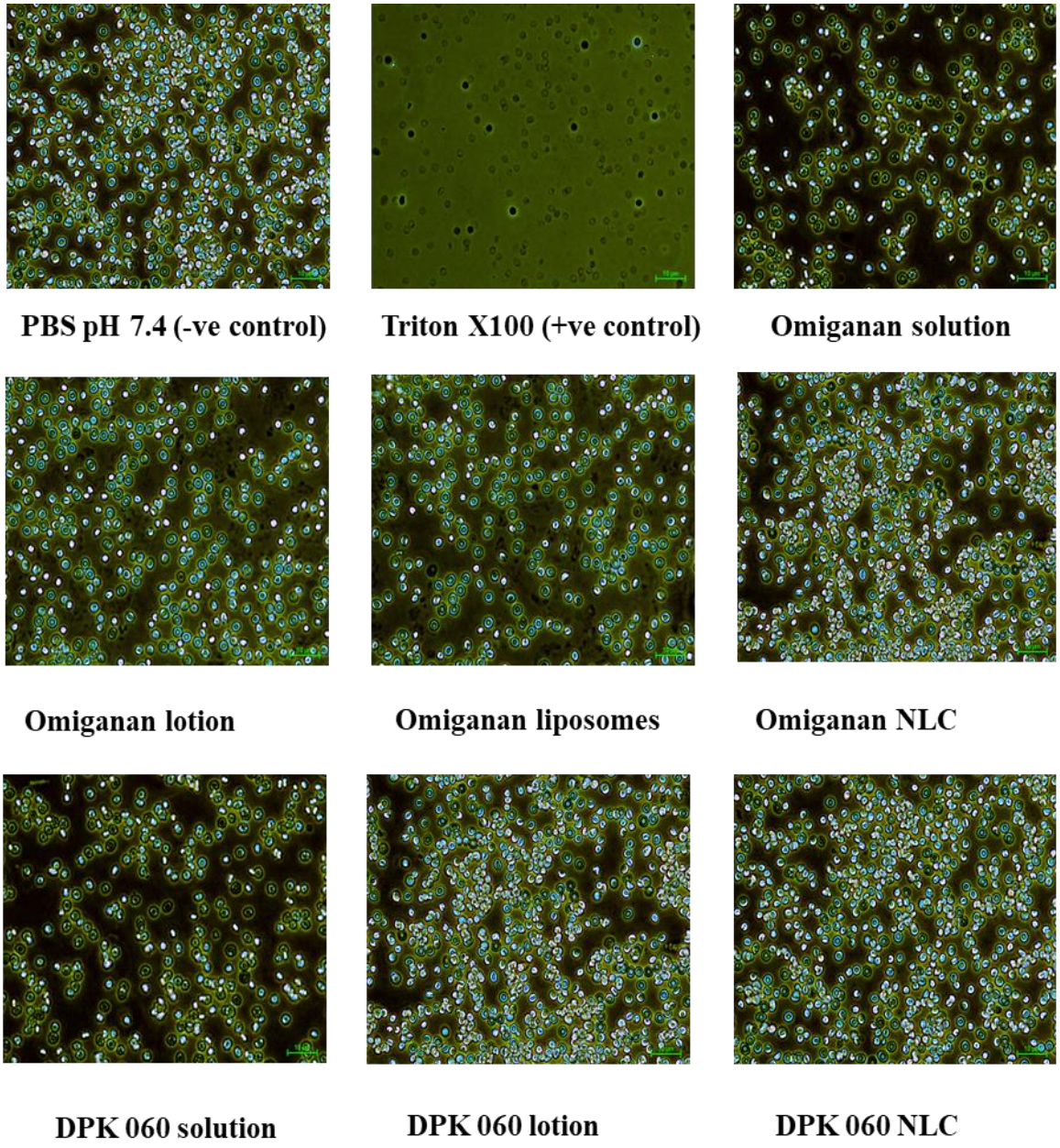


Figure 7.7 Results of hemocompatibility study of Omiganan & DPK 060 formulations

7.4.5 Protease sensitivity assay

The degradation kinetics by the trypsin were examined for Omiganan and DPK-060 loaded formulations to assess the intrinsic susceptibility of Omiganan/DPK-060 at the predefined ratio. Fig. 7.8 and 7.9 display the Omiganan and DPK-060 stability after incubation with trypsin at 20% w/w for Omiganan and 5% w/w for DPK-060. Results with

-ve control (PBS pH 7.4) demonstrated no degradation after incubation at 37°C in PBS pH 7.4 without peptidases. Omiganan (free drug solution and lotion) showed moderate resistance (around 40 % Omiganan was degraded at 20 %w/w trypsin concentration) against the trypsin-induced proteolytic degradation. However, liposomes and NLCs demonstrated higher protection for Omiganan (around 10 % Omiganan was degraded at 20 %w/w trypsin concentration) compared to free drug solution. Whereas DPK-060 (free drug solution and lotion) showed higher susceptibility to trypsin in comparision with Omiganan. 5% w/w of trypsin was adequate for the whole degradation of the DPK 060 after incubation of 4 h. Whereas DPK 060 loaded NLC demonstrated limited but substantial protection against trypsin in comparision to the DPK 060 solution and lotion.

The degradation kinetics by the trypsin were examined for Omiganan loaded formulations to assess the intrinsic susceptibility of Omiganan at a predefined ratio. Data in Fig. 3 display the Omiganan stability after incubation with trypsin at 20% w/w. Results with negative control (PBS pH 7.4) demonstrated no degradation after incubation at 37°C in PBS pH 7.4 without peptidases. Omiganan (free drug solution and lotion) showed moderate resistance (around 40 % Omiganan was degraded at 20 %w/w trypsin concentration) against the trypsin-induced proteolytic degradation. Though, the ability of the formulation to protect Omiganan appears to be higher when Omiganan is entrapped into the liposomes (around 10 % Omiganan was degraded at 20 %w/w trypsin concentration).

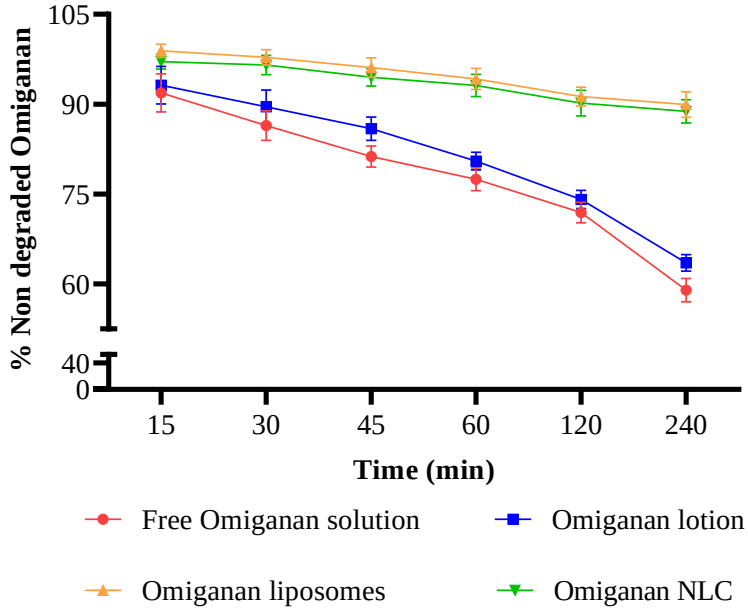


Figure 7.8 Protease degradation assay of Omiganan loaded formulations

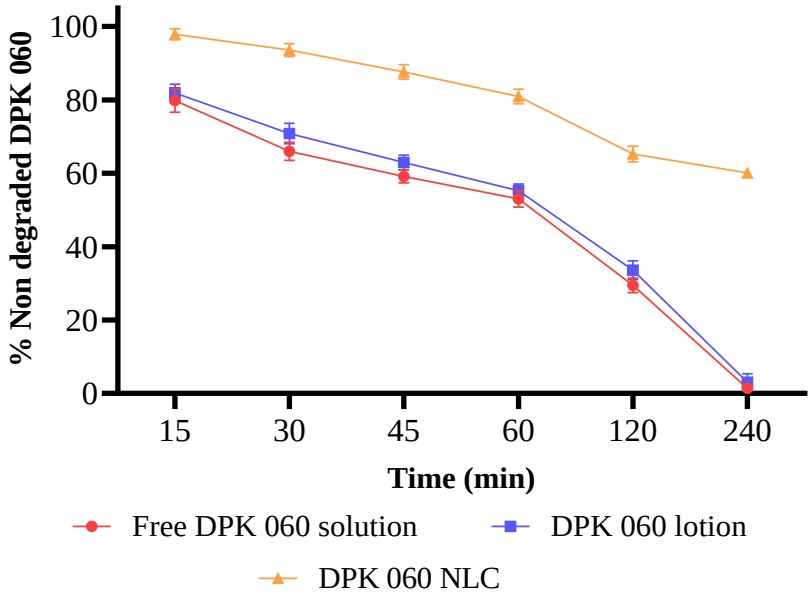


Figure 7.9 Protease degradation assay of DPK 060 loaded formulations

7.4.6 *Ex-vivo* drug permeation and skin retention studies

The results of *ex-vivo* drug permeation and skin retention of Omiganan and DPK 060 loaded formulations are summarized in Tables 7.7 and 7.8, respectively.

Table 7.7 *Ex-vivo* drug permeation and retention of Omiganan loaded formulations

Time (hr)	% Cumulative drug permeation			
	Free Omiganan gel	**Omiganan lotion**	**Omiganan liposomal gel**	**Omiganan NLC gel**
0.5	1.92 ± 1.07	2.47 ± 1.07	5.18 ± 1.42	7.20 ± 1.03
1	3.59 ± 1.26	5.22 ± 1.66	11.71 ± 1.29	13.27 ± 1.31
2	5.33 ± 1.16	7.43 ± 1.81	15.58 ± 1.66	17.15 ± 1.69
3	7.01 ± 1.39	8.72 ± 1.58	19.26 ± 2.34	21.71 ± 2.13
4	9.55 ± 1.18	10.34 ± 1.92	23.04 ± 2.15	26.44 ± 2.61
5	10.87 ± 1.43	11.87 ± 1.35	28.20 ± 2.44	31.05 ± 2.99
6	11.62 ± 2.11	12.90 ± 2.64	31.88 ± 2.65	35.93 ± 2.75
12	12.47 ± 1.85	14.51 ± 2.53	37.19 ± 3.19	42.72 ± 2.49
% Drug retained after 12 h				
--	87.23 ± 3.20	84.08 ± 3.87	61.25 ± 4.08	58.96 ± 3.51

Table 7.8 *Ex-vivo* drug permeation and retention of DPK 060 loaded formulations

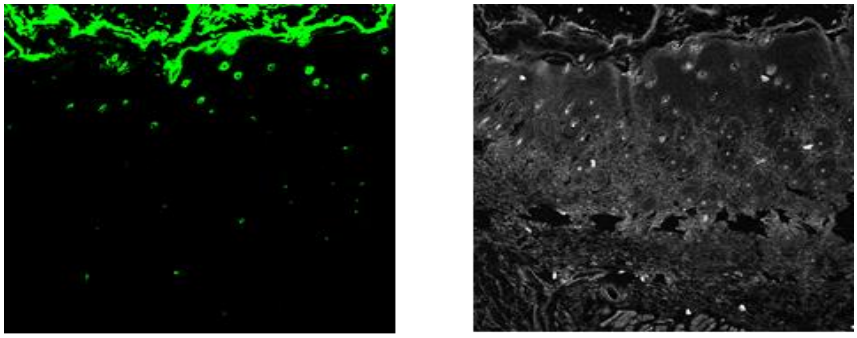
Time (hr)	% Cumulative drug release		
	Free DPK 060 gel	**DPK 060 lotion**	**DPK 060 NLC gel**
0.5	1.20 ± 0.98	2.03 ± 1.15	5.74 ± 1.21
1	2.98 ± 1.34	4.81 ± 1.42	11.23 ± 1.56
2	4.15 ± 1.21	6.50 ± 1.32	14.41 ± 1.92
3	5.82 ± 1.52	7.93 ± 1.71	17.35 ± 2.08
4	7.34 ± 1.40	9.31 ± 1.88	20.57 ± 2.31
5	8.71 ± 1.67	10.97 ± 1.60	23.83 ± 2.67
6	9.66 ± 1.83	12.02 ± 2.16	26.08 ± 2.43
12	11.59 ± 2.10	15.77 ± 2.44	31.28 ± 2.97
% Drug retained after 12 h			
--	89.42 ± 3.65	85.27 ± 3.52	68.13 ± 3.90

The % cumulative amount of drug permeated from Omiganan and DPK 060 nano-formulations after 12 h were found to be 3 folds, respectively higher for NLC based gel when compared to free drug gel and lotion. Additionally, Results demonstrated that around 87% of Omiganan was retained on the skin from the lotion and free Omiganan gel. In contrast, approximately 60% of Omiganan was retained on the skin from the liposomes and NLCs gel. While for DPK 060, results demonstrated that around 89% of DPK 060 was retained on the skin from the lotion and free DPK 060 gel, whereas about 68% of DPK 060 was retained on the skin NLCs gel. The increased permeation of nano-formulations of Omiganan and DPK 060 may be related to the structure and composition of the nano-carriers. Liposomes and NLCs possess a natural affinity for skin and may facilitates transport of drug by enhancing its partitioning from the vehicle to the skin.

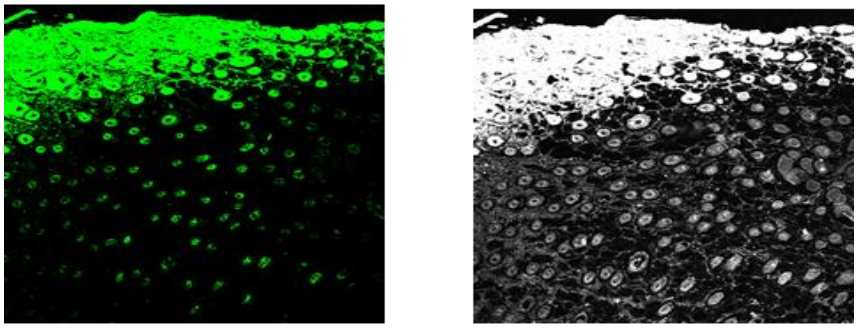
Moreover, the increased permeation of NLC based gel may be due to improved contact time with the skin, sustained release characteristics and an occlusive effect [22]. Despite the hydrophilic behavior of the gel, the high lipid content can lead to an occlusion. Furtermore, film formation and occlusion on skin leads to enhanced skin hydration as a result of reduced trans-epidermal water loss, directs the drug penetration into the SC [22, 23].

7.4.7 *Ex-vivo* fluorescence microscopic study

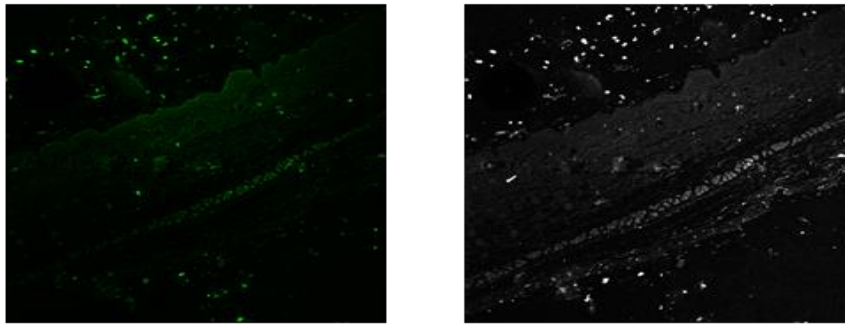
Fluorescence microscopic pictures of mice skin sections after 6 h of treatment with FITC tagged formulations, are demonstrated in Fig. 7.10. and 7.11. Small amount of fluorescence was detected in skin sections treated with free Omiganan and DPK 060 gel and lotion. The formulations can be arranged in the following order of increasing fluorescence for Omiganan formulations: free Omiganan gel<Omiganan lotion<Omiganan liposomal/NLC gel. While for DPK 060 loaded formulations: free DPK 060 gel<DPK 060 lotion<DPK 060 NLC gel. The microscopic fluorescence data were found in line with the *ex-vivo* skin permeation and deposition data, where maximum fluorescence was observed in skin sections treated with Omiganan and DPK 060 loaded NLC gel. The increased permeation of nano-formulations of Omiganan and DPK 060 may be related to the structure and composition of these nanocarriers.



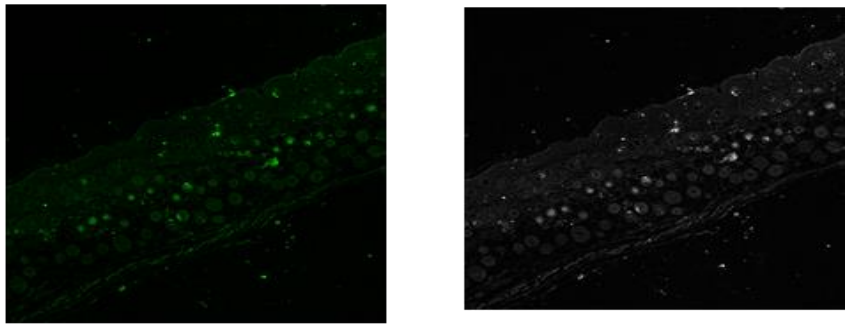
Omiganan Gel



Omiganan Lotion



Omiganan Liposomal gel



Omiganan NLC gel

Figure 7.10 Fluorescence microscopic images of mice skin sections after 6 h of treatment with Omiganan formulations

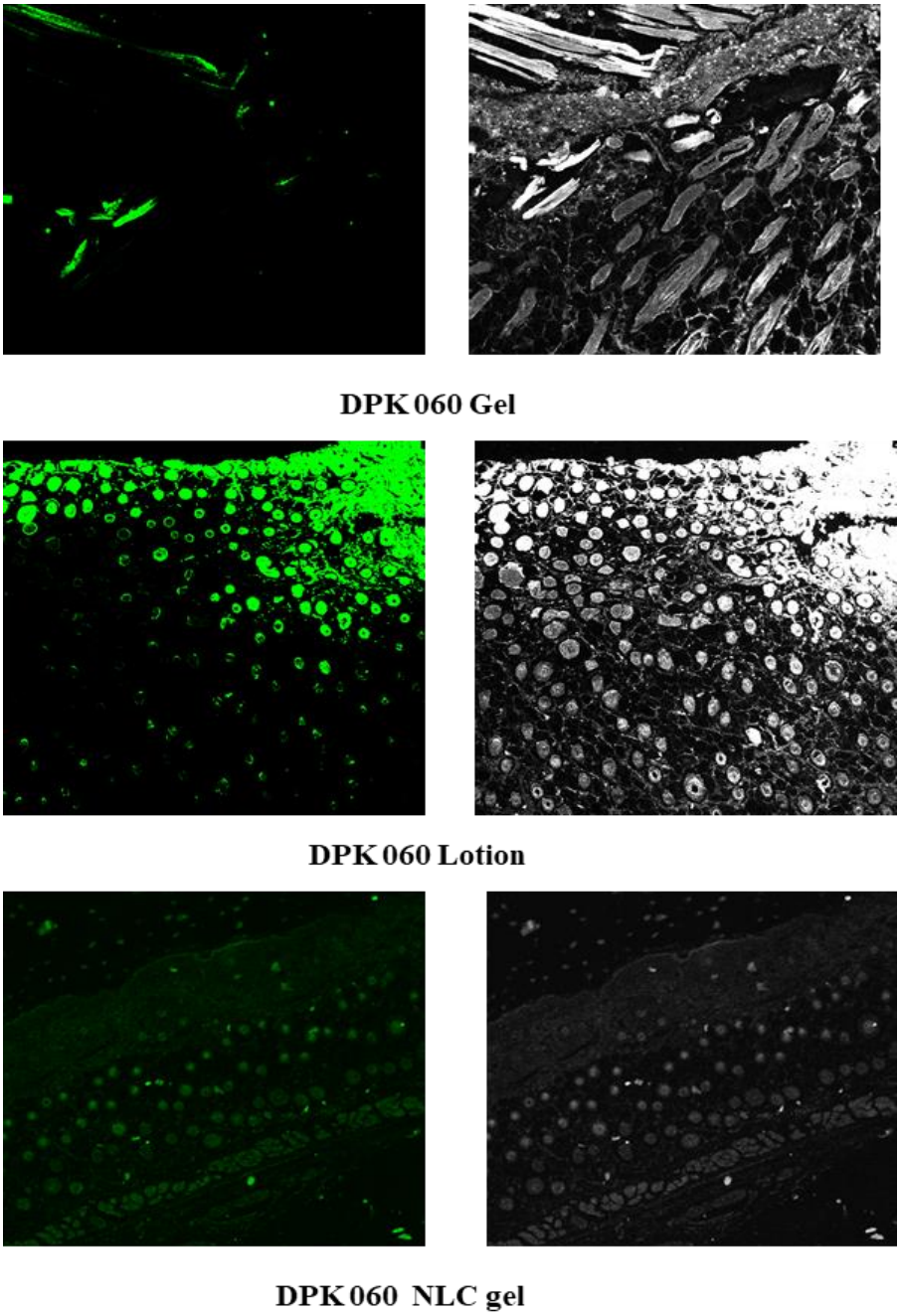


Figure 7.11 Fluorescence microscopic images of mice skin sections after 6 h of treatment with DPK 060 formulations

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