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

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February – 2020

Introduction

Eczema and Psoriasis are the most challenging skin conditions encountered worldwide by skincare professionals. Atopic eczema (AE) is a multifactorial chronic inflammatory skin disease characterized by pruritic, typically distributed eczematous skin lesions. Up to 20% of the children and 1-3% of adults worldwide suffer from eczema (1). Psoriasis is a chronic, immune-mediated, non-communicable, painful, disfiguring, and disabling incurable disease with a great negative impact on patients' quality of life (2). The reported prevalence of psoriasis in developed countries ranges between 0.09% to 11.4% (3), making psoriasis a serious global problem with at least 100 million individuals affected worldwide.

Treatment of eczema and psoriasis are still based on controlling the symptoms. Topical and systemic therapies, as well as phototherapy, are available. In practice, a combination of these methods is often used. The need for treatment is usually lifelong and is aimed at remission. So far, no therapy would cure eczema and psoriasis. The topical treatment of eczema involves emollients, topical corticosteroids (hydrocortisone, clobetasone, and mometasone). While topical treatment of psoriasis involves vitamin D_3 analogs, corticosteroids i.e., betamethasone and hydrocortisone, topical retinoids in the form of a gel, ointments, creams, lotions, and foams while phototherapy (UV light therapy) and use of systemic therapies of methotrexate, cyclosporine, and biological agents (Alefacept, infliximab, adalimumab, ustekinumab) are other treatment approaches. In the treatment of bacterial infections associated with psoriasis and eczema, fusidic acid and some antibacterials were used that had a chance of resistance when applied on a long-term basis (4). The current treatments though manage inflammatory symptoms but have several limitations i.e., corticosteroids can cause thinning of the skin, vitamin D_3 analogs irritate the skin, retinoids do not act as quickly as topical corticosteroids while phototherapy can cause severe and long-lasting burns. Systemic and biological treatments also have limitations i.e. methotrexate can cause liver damage and/or decrease the production of oxygen-carrying RBCs, cyclosporine may provide quick relief of symptoms but the improvement stops when treatment is discontinued, Alefacept can increase the risk of infection possibly including cancer (4).

Psoriasis and eczema can also be provoked by external and internal triggers, including mild trauma, infections, systemic drugs, and stress (5). The results of a randomized clinical trial show that the condition may be worsened by skin/gut colonization with microorganisms i.e., *S. aureus*, Propionibacterium species, *candida albicans*, and some other species (6-8). Specifically, *S. aureus* may induce purulent superinfection and enhance the inflammatory process by superantigen mediated T-cell activation (9). While as in eczema, a skin disease caused by *S. aureus*, decreased the expression of antimicrobial peptides such as cathelicidin LL-37, human beta-defensin 2, and human beta-defensin 3 (10-12).

Protein and peptides in the biological system form an important class of therapeutics currently getting investigated for various disorders such as cancer, diabetes, immune diseases, brain disorders, etc. The potential benefits of protein and peptide antimicrobials for dermal use are their no or reduced chances of development of resistance, broad spectrum of anti-microbial activity with almost no side effects observed in conventional therapy, and may also have anti-inflammatory activity (13, 14). Delivery of these classes of drugs through the dermal route remains challenging due to conformational instability, low permeability across stratum corneum, partitioning in a different stage of the subdermal region, enzymatic degradation, etc. (15). Dermal route for delivery of therapeutics has been continuously investigated to supplement the limitation of oral and other routes of drug delivery for skin disorders. There are limitations of delivering protein and peptide antimicrobial based therapeutics orally due to the harsh environment of the gastrointestinal tract. Delivery via the intravenous route, intramuscular, intra-peritoneal routes display higher bioavailability, are invasive ones and limit patient compliance, and are of little benefit in the treatment of skin disorders. The dermal route of delivery is non-invasive and offers the advantage of ease of application. (15).

Carriers of protein and peptide therapeutics may involve the use of lipidic and polymeric vectors that are a diverse class of delivery systems having biocompatibility. Liposomes are lipid vesicles having single or multiple lipid bilayers composed of mixtures of phosphatidylcholines with long or short hydrocarbon chains and can be designed to suit the need to achieve various morphological states based on the processing conditions such as hydration, temperature, and composition (16, 17). Other delivery systems investigated

for enhanced skin permeation include Transferosomes, Ethosomes, Niosomes (18), Microemulsions, and solid-lipid nanoparticles (19-21). Polymeric vectors such as nanoparticles composed of natural [chitosan, (22)] and synthetic polymers [polyalkylcyanoacylates, poly-lactic acid (PLA), poly-ε-caprolactone (PCL), poly-glycolic acid (PGA)] have been investigated (23, 24). Thus, a wide variety of delivery systems have been investigated and can further be explored for delivering the therapeutics successfully via the dermal route.

Lipid based nano-carriers such as liposomes, SLN and NLCs have a natural affinity for skin lipids. This character facilitates drug transport by increasing its partitioning from the vehicle to the skin. Moreover, they offers enhanced permeation of drug(s), may be attributed to increased contact with the skin layer, an occlusive effect, and sustained release properties (25). Also in the case of hydrophilic molecules, the high lipid content can lead to an occlusive effect and film formation on skin, lead to increased skin hydration due to reduced trans-epidermal water loss, promoting the penetration of drugs into the SC (25, 26).

In addition to this, lotion-based formulation approach has several advantages such as avoidance of first pass metabolism, easy to apply, improved patient compliance, improved physiological and pharmacological response, avoidance of gastrointestinal incompatibility and ability to easily terminate medications when needed (27). Lotions are usually suspensions of solids in an aqueous medium. It is a low to medium viscosity topical preparation and are thicker and emollient in nature in comparison to solution. They are usually oil mixed with water, and more often than not have less alcohol than solutions (27).

In a nutshell, this research proposal aims to investigate the conventional (lotion) and nanotechnology-based approaches to overcome the challenges of conformational instability, low permeability across stratum corneum, partitioning in a different stage of the subdermal region and proteolytic degradation and develop suitable dermal drug delivery products.

OBJECTIVE:

The prime objective of the study is to formulate efficient delivery systems to deliver therapeutic protein/peptide to combat bacterial infections associated with eczema and psoriasis resulting in improved efficiency and a better treatment approach.

The proposed research aims to:

- Formulation of antimicrobial peptide-loaded nano-carrier(s) (liposomes and nano-lipid constructs)
- Characterization and evaluation of the designed protein/peptide(s) delivery system for their physicochemical parameters and in vitro release characteristics.
- Evaluation of cell uptake and toxicity of the designed delivery system by cell line studies.
- Study in vivo performance of the designed protein/peptide(s) delivery system for their efficacy using the suitable animal model.

Antimicrobial Peptides: Omiganan and DPK-060

❖ Omiganan:

Omiganan is a novel, synthetic, cationic peptide and an analog of indolicidin, a 12 amino acid short AMP of the cathelicidin family. It has demonstrated antimicrobial activity against a wide variety of gram-positive and -negative bacteria and fungi (28, 29). Apart from its antimicrobial effect, Omiganan also has anti-inflammatory activity (30). These, antimicrobial and anti-inflammatory properties make Omiganan a promising agent for the treatment of eczema/atopic dermatitis and psoriasis. Furthermore, the positive phase II clinical results were obtained in patients with mild to moderate atopic dermatitis with Omiganan 1% gel (31). This study applies new formulation strategies i.e., lotion, liposomes, and Nano-lipid constructs of Omiganan for dermal delivery, and characterizes these different formulations in terms of preclinical in vitro, ex vivo, and in vivo studies.

- Omiganan was synthesized by S-Biochem, Kerala, India. The detailed sequence of Omiganan is mentioned below:
  - 12- amino acid sequence (ILRWPWWPWRRK-NH2)
  - Molecular weight: 1779.2 gm/mole

❖ **HPLC Method Development for Omiganan:**

❖ **Gradient HPLC method:**

Instrument: Agilent gradient HPLC

Reagents/Solvents: Acetonitrile HPLC Grade, Trifluoracetic acid, Milli Q Water

Gradient Chromatographic conditions:

Parameters	**Chromatographic Conditions**
Mobile Phase	Mobile Phase A: Acetonitrile with 0.1% Tri fluro acetic acid (TFA) Mobile Phase B: Milli Q water with 0.1% TFA.
HPLC column	C18 Column, 4.6 µm,250 mm (Thermo Scientific)
UV wavelength	220 nm
Injection volume	20µl
Flow Rate	1 ml/min
Run Time	30 min

Gradient Programme for Omiganan:

Time (Min)	**Pump A**	**Pump B**	**Flow Rate (ml/min.)**
0.0 – 25.0	32%	68%	1
25.0 – 25.1	57%	43%	1
25.1 – 30.0	100%	0%	1
30.0	Stop		

Preparation of Calibration plot of Omiganan:

Accurately weighed 10.0 mg Omiganan was transferred to a 10 ml capacity volumetric flask and dissolved in Milli Q water, and the volume was made up with Milli Q water (1 mg/ml). This solution was further diluted to make a 100 µg/ml concentration of Omiganan. (Dilution was made with Milli Q water). Subsequently, the calibration plot of the Omiganan was carried out in the range of 1-5 µg/ml.

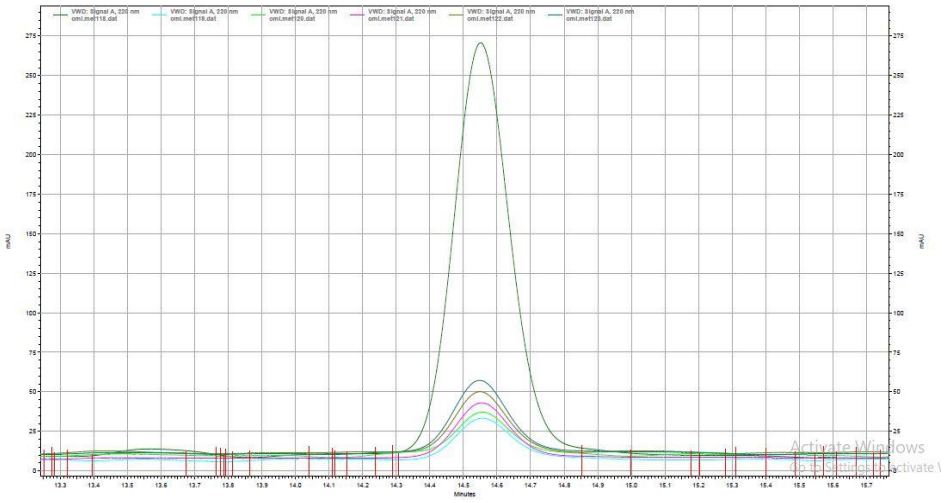

Figure 1a) HPLC overlay chromatogram of Omiganan

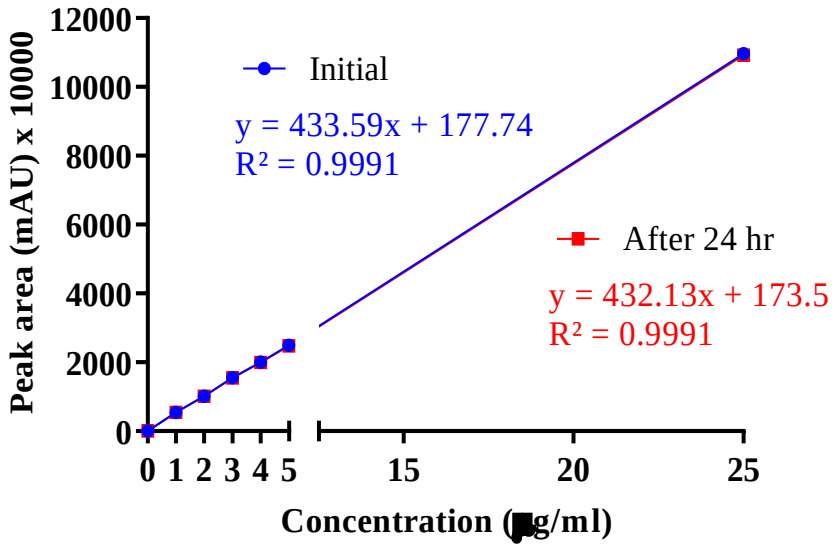

Figure 1b) Calibration plot of Omiganan by HPLC method

Solid-state analysis (FTIR): FTIR results (figure 2) indicates the excipient compatibility between the Omiganan and the physical mixture of Omiganan.

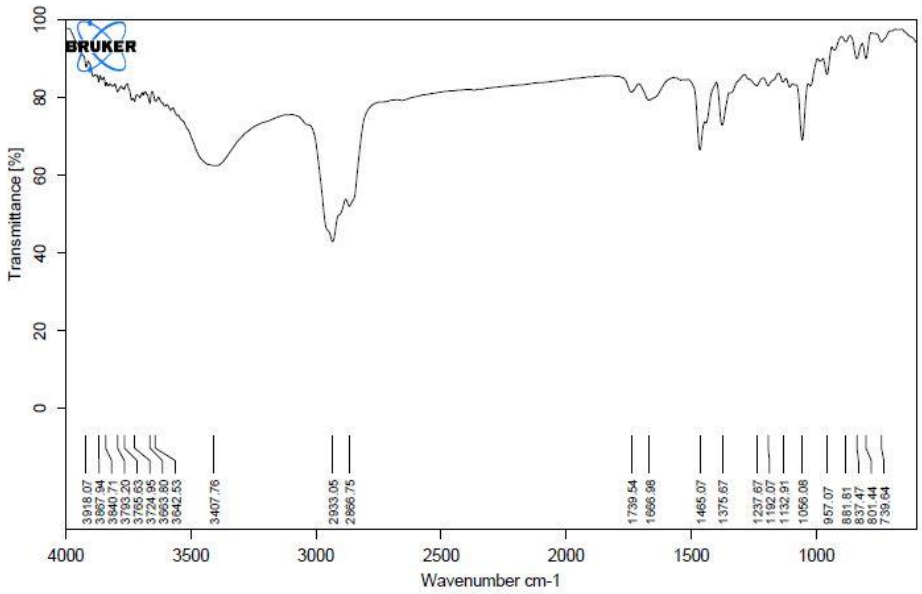

Figure 2 a) FTIR spectra of Omiganan

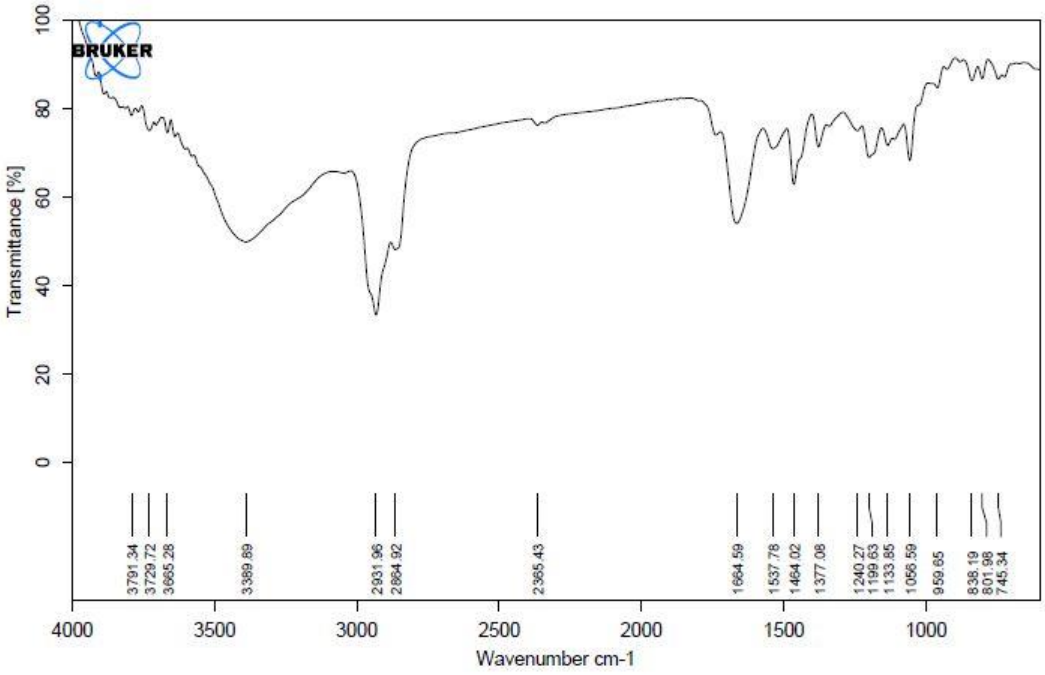

Figure 2 b) FTIR spectra of Omiganan physical mixture (Omiganan+ Precirol-ATO-5 + Soya lecithin + DPPC + HSPC + Cholesterol) (Liposomes/NLC)

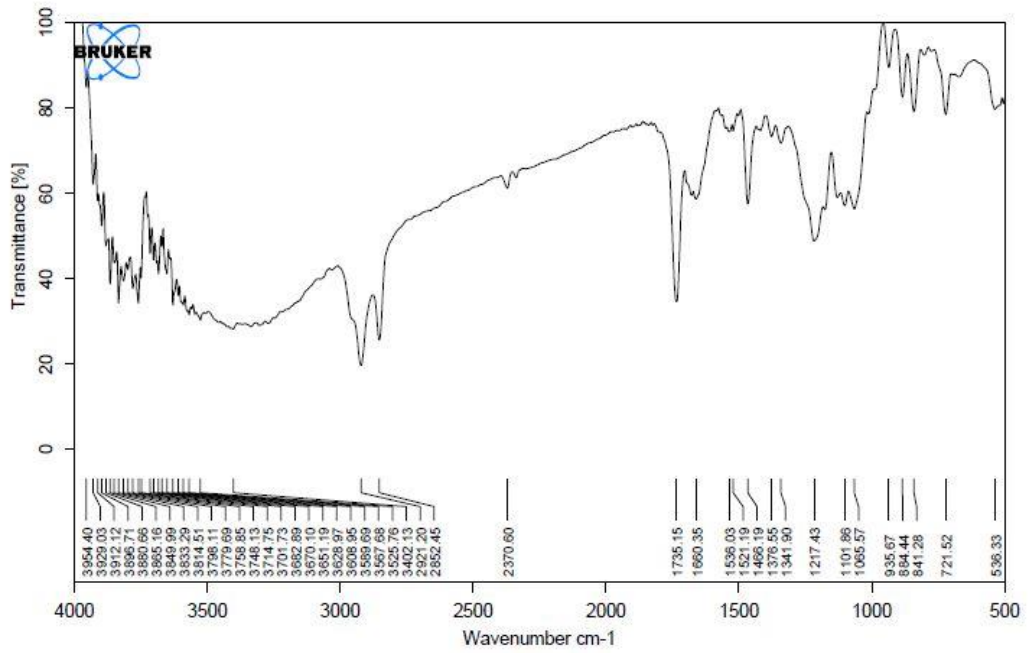

Figure 2c) FT-IR spectra of Omiganan and lipids mixture (Omiganan + Ceteareth-25 + Steareth-10 + PVP K 30) (for lotion)

- **<u>Omiganan Lotion (1%) Formulation:</u>**

- Different excipients were screened thoroughly for the preparation of a lotion-based formulation of the Omiganan. The optimized batch of the formulation having the following excipients (due to the better compatibility and no interference, were selected for the preparation of the optimized batch).

Optimized Batch: Omiganan (1%) lotion

Ingredients	%Quantity (%w/w)
Omiganan (1%)	1%
Steareth-10	0.3%
Cetyl alcohol	4%
Ceteareth-25	2.5%
Isopropyl Myristate	5%
Propyl Paraben	0.02%
Propylene Glycol	5%
PVP K30	2%
Methyl Paraben	0.1%
Phosphate Buffer (PBS) pH 5.5	Q.S to 100 %

Method of Preparation (Peptide-based Lotion)

1. The oil phase was prepared by mixing weighed quantity of cetyl Alcohol, steareth-10, ceteareth-25, and isopropyl myristate and heating at 70°C.
2. The aqueous phase was prepared by dissolving weighed quantity of Omiganan and PVP K 30 in sufficient volume of PBS pH 5.5.
3. Methylparaben and propylparaben were dissolved in an appropriate volume of PBS pH 5.5 and after that propylene glycol was added and this mixture was added into the solution obtained in step 2.
4. The remaining quantity of PBS pH 5.5 was added to the aqueous phase and heated at 70°C.
5. The heated oil Phase was added into the heated aqueous phase and mixed with stirring using a magnetic stirrer. Further, the formed lotion Formulation was cooled to room temperature and filled in the tube. (at the same temperature).

❖ **Omiganan Liposomes (1%):**

Several methods reported in the literature were tried including thin-film hydration, ethanol injection, reverse-phase evaporation, and double emulsion. From these methods, the reverse-phase evaporation method was found to produce liposomes of desired characteristics. Briefly, the lipid phase was prepared by dissolving all lipids (HSPC, DPPC, soya-lecithin, and cholesterol) in chloroform in the required quantity in a glass vial under continuous stirring at 1200 rpm using a magnetic stirrer at room temperature. Subsequently, an accurately weighed quantity of Omiganan was dissolved in PBS pH 5.5 and was added to the lipid phase under continuous stirring using a magnetic stirrer at 1200 rpm at room temperature to form a water-in-oil emulsion. The obtained multi-lamellar liposomal dispersion was then subjected to probe sonication for size reduction.

Optimized Batch: Omiganan Liposomes

Parameter	Optimized value
Lipid	HSPC, DPPC, soya-lecithin, Cholesterol
Internal Lipid Ratio	5.5:3:1:0.5
Stirring speed	1200 rpm
Stirring time	2 hr
Organic phase volume	2 ml
Omiganan: Lipid	1:4 (molar ratio) 1:10 (weight ratio)

From these preliminary examinations, the factorial design was used for further optimization based on the formulation variables and process parameters that may affect the response variables i.e., vesicle size, PDI and % drug entrapment, etc.

❖ Characterization of the Omiganan Liposomes:

Vesicle size, zeta potential, and % entrapment efficiency:

- Vesicle size and zeta potential of the optimized formulation were found to be 120 nm and -17.2 mv respectively and are showed in Figures 3 and 4. % drug entrapment of the peptide in the formulation was found to be 72 % with a loading capacity of 7.8%.
- To determine the % drug entrapment, Omiganan liposomal dispersion was passed through a Sephadex G-50 column. The separated liposomal fraction was dissolved in water: methanol (2:8) mixture and analyzed by developed HPLC method. To ensure the mass balance of the drug, the supernatant was also analyzed for the free drug content using the HPLC method at 220 nm. The % drug entrapment and % drug loading were calculated by using the following equations: (32)

$$\%\ \boldsymbol{Entrapment\ efficiency} = \frac{\boldsymbol{Amount\ of\ Entrapped\ drug}}{\boldsymbol{Total\ drug\ added}}\ \boldsymbol{x\ 100}$$

$$\%\ \boldsymbol{Drug\ loading} = \frac{\boldsymbol{Drug\ loaded\ (mg)}}{\boldsymbol{Total\ weight\ of\ liposomes\ (mg)}}\ \boldsymbol{x\ 100}$$

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 120.5	Peak 1:	142.5	100.0	68.71
PdI: 0.190	Peak 2:	0.000	0.0	0.000
Intercept: 0.957	Peak 3:	0.000	0.0	0.000
Result quality : Good				

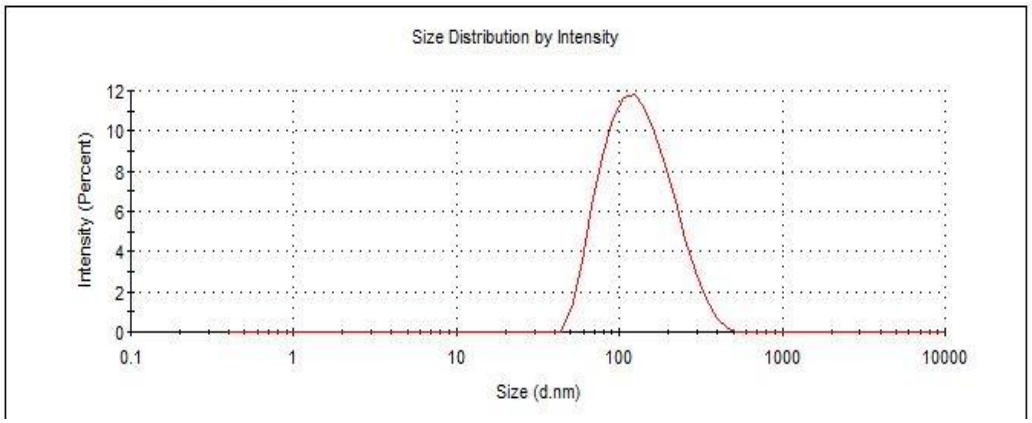

Figure 3 Vesicle size of the developed Omiganan liposomes

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -17.2	Peak 1:	-17.7	100.0	5.99
Zeta Deviation (mV): 5.99	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.154	Peak 3:	0.00	0.0	0.00
Result quality Good				

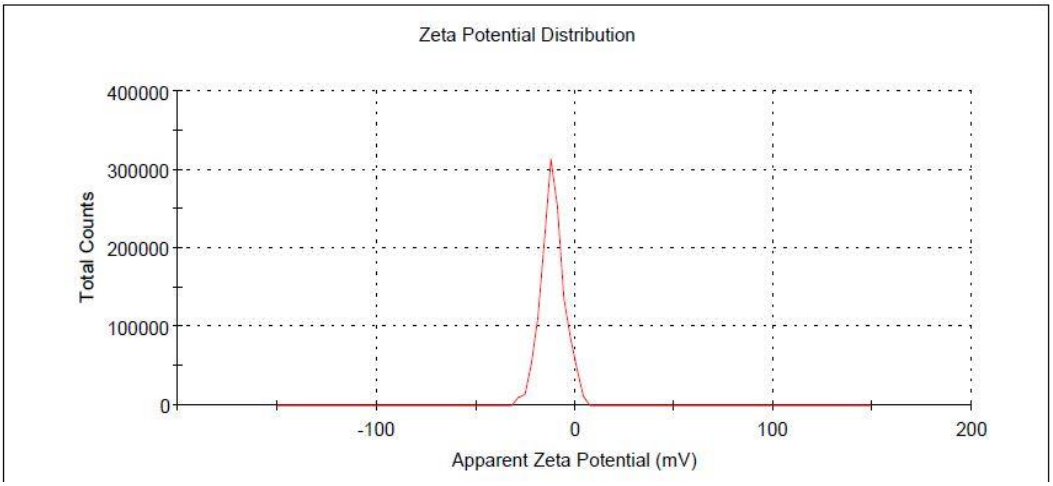

Figure 4 Zeta potential of the developed Omiganan liposomes

❖ **Cryo-Transmission Electron Microscopy (Cryo-TEM):**

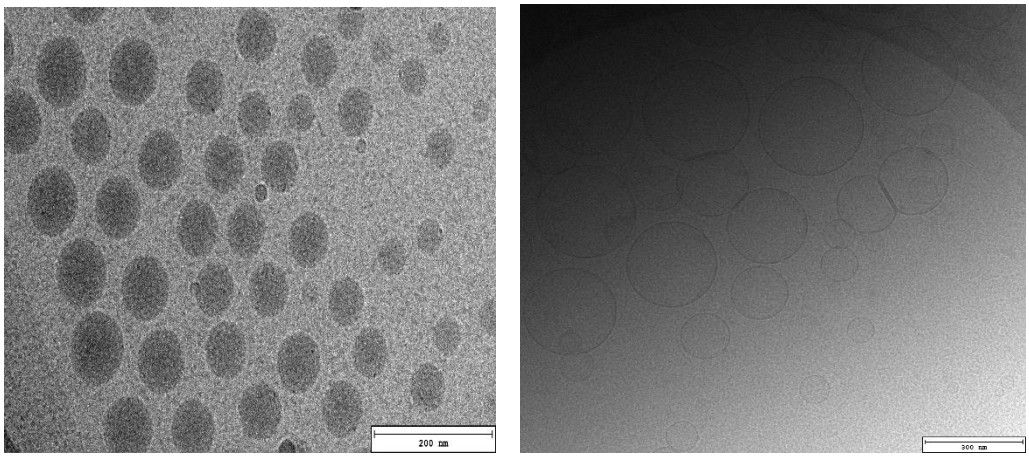

Figure 5 Cryo-TEM images of the developed Omiganan loaded liposomes

❖ **<u>Omiganan Nano-lipid constructs (1%):</u>**

Omiganan loaded nano-lipid constructs (NLCs) were prepared by using melt-emulsification – high-pressure homogenization technique. Briefly, solid lipid and liquid lipid were taken in 6:4 ratios (% w/w). The aqueous phase was prepared by dissolving the surfactant and co-surfactant in 5 ml of PBS pH 5.5. Both of these phases (lipid and aqueous) were maintained at 75^{o}C and Omiganan was added into the melted lipid matrix, followed by addition of the aqueous phase to the lipid phase drop by drop while continuous stirring at 700 rpm to get a homogeneous emulsion. The obtained primary emulsion was then subjected to high-pressure homogenization (10000 psi, 10 cycles) for size reduction.

Optimized Batch: Omiganan Nano-lipid constructs

Parameter	Optimized value
Lipids	Precirol-ATO-5 (solid lipid), Miglyol- 840 (liquid lipid), Tween 80, and PEG 400 (surfactant and co-surfactant)
Solid: Liquid Lipid Ratio	6:4 (% w/w)
Stirring speed	700 rpm
HPH parameters	10000 psi, 10 cycles
Omiganan: Lipid	1:3

❖ Characterization of the Omiganan Nano-lipid constructs:

Particle size, zeta potential, and % drug entrapment:

- Particle size and zeta potential of the optimized formulation were found to be 115 nm and -16.1 mv respectively and were showed in Figures 6 and 7. % Drug entrapment of the peptide in the formulation was found to be 79 % with a loading capacity of 12.7%.
- To determine the % drug entrapment, Omiganan NLC dispersion was centrifuged at 18,000 rpm (4 °C for 30 min). The separated NLC fraction was dissolved in water: methanol (2:8) mixture and analyzed by developed HPLC method. To ensure the mass balance of the drug, the supernatant was also analyzed for the free drug content using the HPLC method at 220 nm. The % entrapment efficiency and % drug loading were calculated by using the following equations: (32)

$$\%\ \boldsymbol{Drug\ entrapment} = \frac{\boldsymbol{Amount\ of\ entrapped\ drug}}{\boldsymbol{Total\ drug\ added}}\ \boldsymbol{x\ 100}$$

$$\%\ \boldsymbol{Drug\ loading} = \frac{\boldsymbol{Drug\ loaded\ (mg)}}{\boldsymbol{Total\ weight\ of\ NLCs\ (mg)}}\ \boldsymbol{x\ 100}$$

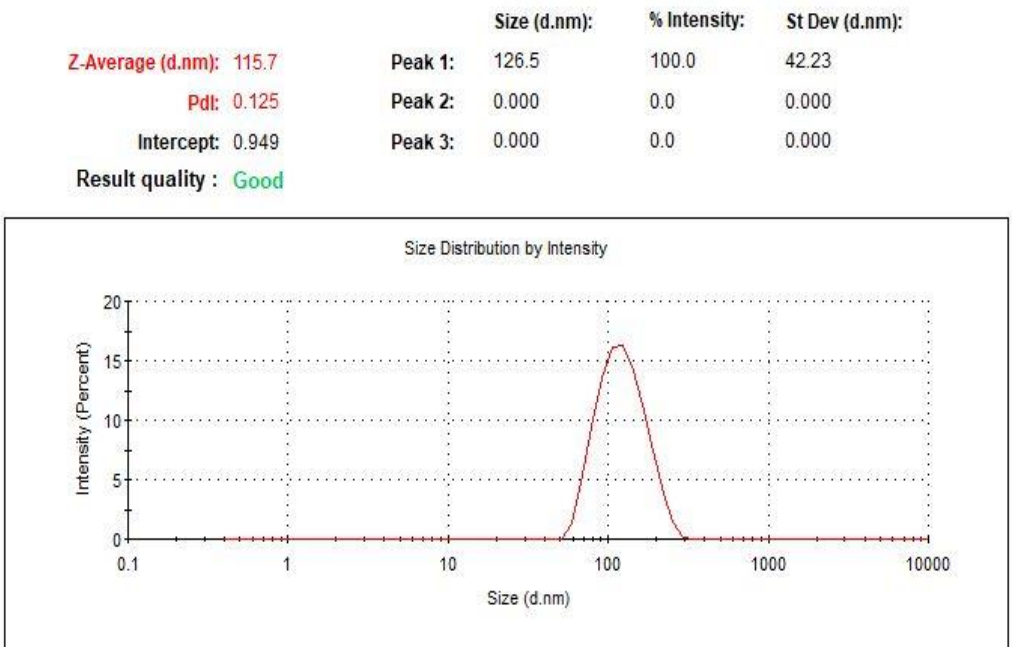

Figure 6 Particle size of the developed Omiganan NLCs

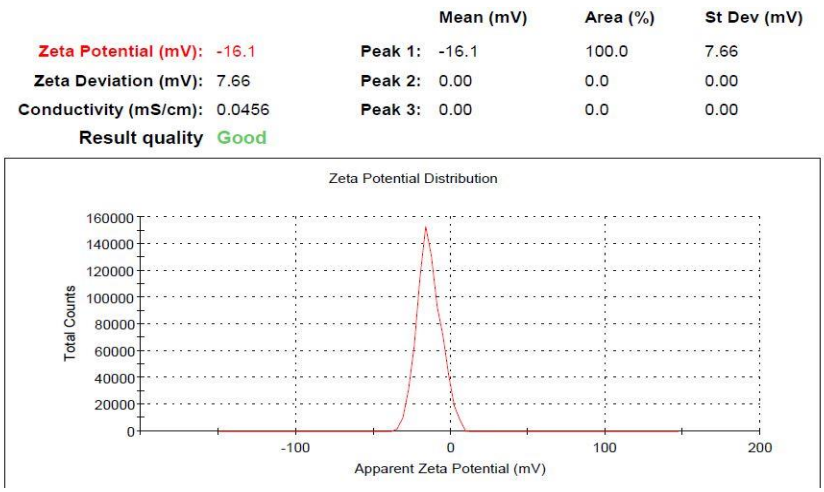

Figure 7 Zeta potential of the developed Omiganan NLCs

- **Scanning Electron Microscopy (SEM):**

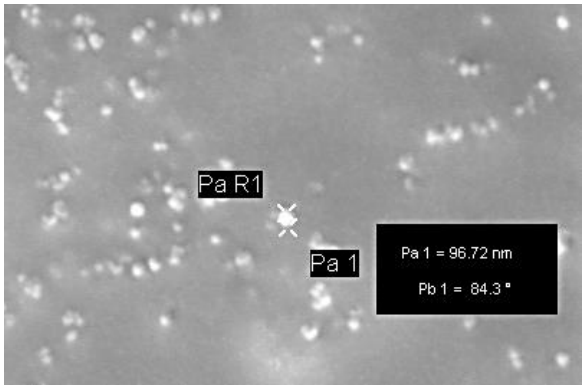

Figure 8 SEM image of the developed Omiganan loaded NLCs

- **Carbopol based gel was formulated as a vehicle for the delivery of Omiganan liposomes and NLCs.**
    - The gel-based formulation was developed and loaded with Omiganan (1%w/v) and optimized Omiganan liposomes/NLCs (1%w/v) to improve the viscosity of the formulation in order to enhance the retention on the skin surface. Carbopol 934P polyacrylic acid-based gelling agent is extensively used in topical formulations. Due to the better hydration and wetting properties, Carbopol 934P requires very less hydration time and also forms uniform dispersion. Briefly, weighed quantity of Carbopol 934P (1.2%w/v) was mixed and dispersed in Omiganan liposomal/NLC dispersion (1%w/v) using an overhead stirrer at 2000 rpm for 1 h. After complete hydration, the mixture of

preservatives namely methylparaben (0.2%w/v) and propylparaben (0.02%w/v) in propylene glycol (4%w/v) was added with continuous stirring, and the pH was adjusted to ~6.5 by dropwise addition of 10% v/v Sodium hydroxide solution. The detailed formulation composition is shown in the following table.

Formulation components	Concentration
Omiganan liposomes/NLCs	1% w/v
Carbopol 934P	1.20% w/v
Propylene glycol	4% w/v
Methyl paraben, propyl paraben	0.2 and 0.02 %w/v
Sodium hydroxide solution (10%)	…qs… (pH adjustment of 6.5)

❖ **Characterization of Omiganan gel:**

❖ **Viscosity of Omiganan gel**

The viscosity of free Omiganan gel, Omiganan liposomal, and Omiganan NLC gel was measured using cone and plate rheometer (Bohlin C-VOR, Malvern Instruments Ltd., Worcestershire, UK) at 25±1ºC. Briefly, 200 mg of the gel sample was placed on the sample holder and the spindle (plate with 20 mm diameter and 4º cone angle) was lowered and allowed to equilibrate for 5 min. The spindle was rotated at a shear rate of 10/s and corresponding viscosity (Pa.S) was reported (33).

Results: The viscosities of free Omiganan gel, Omiganan liposomal, and Omiganan NLC gel were found to be 12.49 ± 0.367 Pa.S, 14.05 ± 0.420 Pa.S, and 15.42 ± 0.37 Pa.S respectively.

❖ **Spreadability of Omiganan gel**

The spreadability of free Omiganna gel, Omiganan liposomal, and Omiganan NLC gel was evaluated by the reported method (34). Briefly, 500 mg of gel sample was positioned within the circle of 1 cm diameter pre-marked glass plate over which second glass plate was placed. The 500 g weight was applied on the upper glass plate for 5 min and an increase in diameter was noted.

Results: The spreadability of free Omiganna gel, Omiganan liposomal, and Omiganan NLC gel was found to be 6.21 ± 2.34 cm^2, 7.39 ± 2.49 $cm^{2,}$ and 8.32 ± 1.85 cm^2 respectively. The spreadability of free Omiganna gel was lesser than Omiganna liposomal/NLC gel which may

be attributed to the increased solid content of the gel after the addition of liposomal/NLC formulation.

❖ **pH of Omiganan gel**

The pH of free Omiganna gel, Omiganan liposomal, and Omiganan NLC gel was determined using a digital pH meter (Lab India Pvt. Ltd, Mumbai). A pH measurement of the samples was performed in triplicate, and the average value was reported.

Results: The pH of free Omiganan gel, Omiganan liposomal, and Omiganan NLC gel were found to be 6.4 ± 0.3, 6.5 ± 0.4, and 6.6 ± 0.3 respectively.

❖ **In-vitro release studies:**

In vitro release of Omiganan from lotion, liposomes, and NLCs was carried out using a widely used dialysis bag technique. Dialysis bag (Mole. weight - 12 KDa cut-off) containing the free Omiganan gel (1%), Omiganan lotion (1%), Omiganan liposomal (1%), and NLCs gel (1%) were placed in a 30 mL of release media (PBS pH 7.4). Samples (0.5 ml) were withdrawn at regular time intervals (0.5,1,2,3,4,5,6 and 12 hr) from the receptor compartment and the same volume (0.5 ml) was replaced by a fresh diffusion medium. The samples were analyzed using the developed HPLC method as described above. All the experiments were performed in triplicate and the average values were taken.

Results: Results demonstrated that around 92% of Omiganan was released from the lotion and free Omiganan gel within the 1hr whereas around 77% of Omiganan was released from the liposomes and NLCs after 6 hr, offers the sustained release of Omiganan. The quick equilibrium was observed from lotion and free Omiganan gel due to its hydrophilic nature.

❖ **Ex-vivo permeation and skin retention studies:**

Ex-vivo permeation was carried out 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 Franz diffusion cell with a diameter of 9 mm, and free Omiganan gel (1%), Omiganan lotion (1%), Omiganan liposomal (1%), and NLCs gel (1%) was added to the donor compartment. The assay conditions were the same as the in the vitro release section. After 12 h, the skin samples

were removed from the diffusion cell, and the surface was thoroughly washed with PBS pH 7.4 and cut into small pieces, and then added to a tube containing 5.0 mL of Ethanol for homogenization. All samples were vortexed for 1 min and the Omiganan content was quantified by HPLC.

Results: Results demonstrated that around 87% of Omiganan was retained on the skin from the lotion and free Omiganan gel whereas around 60% of Omiganan was retained on the skin from the liposomes and NLCs gel indicating better penetration from the developed NLCs.

❖ **In-vitro MTT assay:**

A study was performed in the 3T3-fibroblast cells to assess the cytotoxicity of the developed formulations. Cells were seeded with 5×10^3 cells/well cell density in a 96 well microtiter plate using DMEM supplemented with 10% Fetal Bovine Serum. 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, the cells were exposed at different w/w ratio and evaluated for 6 h. After the exposure time, cell media was replaced with a complete medium containing 1% solution of antibiotic and 10% Fetal Bovine Serum. After 24 h, cells were washed with PBS pH 7.4, and MTT dye (5mg/mL) solution (20 µl) was added to each well plate. The MTT dye was permitted to react for 4 h under incubator condition, after that cell medium in each plate was replaced with 100 µl of DMSO (Himedia, Mumbai) and the microtiter plate was shaken gently to dissolve the crystals of formazan. The color of the formazan was determined using a Biorad microtiter plate reader (Biorad, California) at 570 nm. PBS treated Cells were used as control cells. The absorbance values of cells treated with PBS were taken as 100% cell viability and all other treatments were expressed relative to it (35, 36). Omiganan in the concentration range of 0.01 – 100 µg/ml was evaluated for the MTT assay with Triton X 100 and PBS pH 7.4 as positive and negative control respectively.

Results: The viability of cells treated with free Omiganan, 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%). The developed Omiganan liposomes/NLCs have no potential toxic effects on the 3T3-fibroblast cells and are found to be safe for dermal delivery.

❖ **Cellular Uptake by Confocal Microscopy:**

The in vitro cell-based assays were carried out on 3T3-fibroblast cells. Cells were seeded with 1×10^5 cells/well cell density in a 6 well plate using DMEM supplemented with 10% Fetal Bovine Serum. The cell culture was grown for 24 hrs in a CO_2 incubator maintained at 5%concentration and humidified with saturated Copper sulfate solution. To evaluate the cellular uptake, FITC tagged Omiganan was loaded in the formulations. Briefly, cells were seeded at a cell density of 1×10^5 cells/well in 6-well cell culture plates, allowed to adhere for 24 h followed by treatment with FITC tagged Omiganan formulations. The cellular uptake was determined at 1 and 4 h treatment by confocal microscopy.

Results: The cellular uptake of the different Omiganan formulations are depicted in figure 9. Results demonstrated the higher cellular uptake of the Omiganan loaded liposomes and NLCs in comparison to free Omiganan gel and Omiganan lotion after 4 h, which results in the enhanced therapeutic efficacy of the developed formulations (liposomes/NLCs).

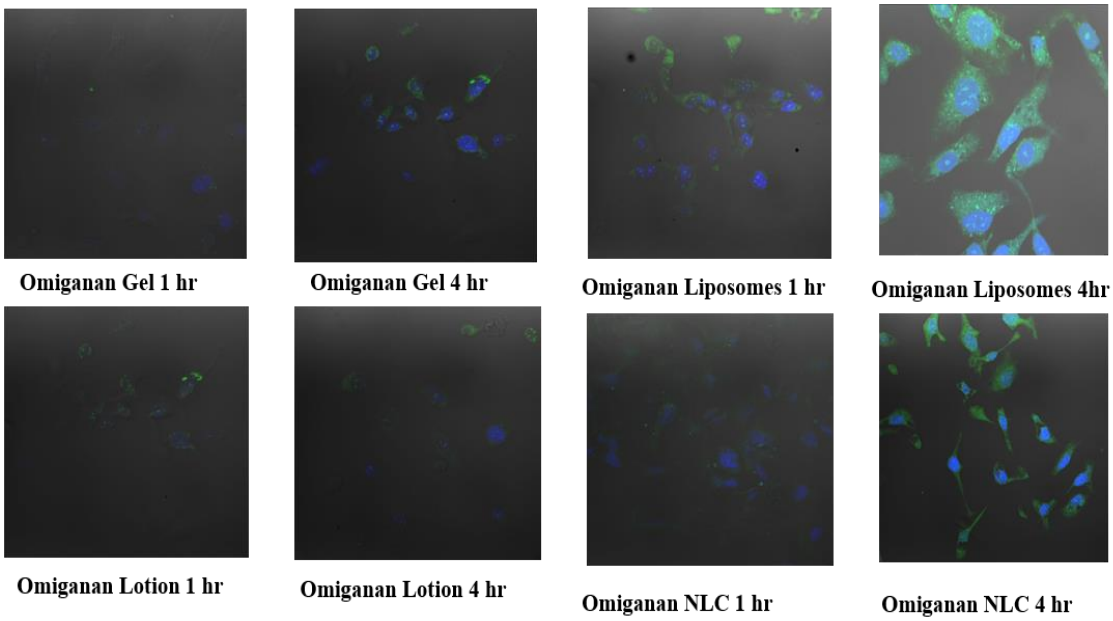

Figure 9 Cellular uptake of Omiganan formulations

❖ DPK 060:

- DPK-060 is a chemically synthesized peptide structurally derived from human protein kininogen, where three tryptophan residues have been added to the C-terminal end of the endogenous 17-amino acid sequence (37). In comparison to its endogenous analog, this addition enhances the ability of DPK-060 to withstand enzymatic degradation by infection-affiliated proteases, without any signs of aggravated cytotoxicity (38, 39). DPK-060 exhibits a potent broad-spectrum antimicrobial activity in vitro against both Gram-positive and Gram-negative bacteria including methicillin-resistant Staphylococcus aureus (MRSA) (40). The safety and efficacy of 1% DPK-060 in a polyethylene glycol (PEG)-based ointment has been studied in a clinical phase II trial (NCT01522391) in the treatment of skin infections in atopic dermatitis patients. The results of this trial revealed that DPK-060 1% ointment was well tolerated by the subjects and significantly reduced the microbial density in eczematous lesions after 14 days with twice daily application compared with placebo. This study applies new formulation strategies i.e., lotion and Nano-lipid constructs of DPK-060 for dermal delivery, and characterizes these different dose formulations in terms of preclinical in vitro, ex vivo, and in vivo studies.
- DPK-060 was synthesized by S-Biochem, Kerala, India. The detailed sequence of DPK 060 is:
  - 17- amino acid sequence (GKHKNKGKKNGKHNGWKWWW)
  - Molecular weight: 2.5 kDa

❖ HPLC Method Development for DPK-060:

❖ Gradient HPLC method:

Instrument: Agilent gradient HPLC

Reagents/Solvents: Acetonitrile HPLC Grade, Trifluoracetic acid, Milli Q Water

Gradient Chromatographic conditions:

Parameters	**Chromatographic Conditions**
Mobile Phase	Mobile Phase A: Acetonitrile with 0.1% TFA. Mobile Phase B: Milli Q water with 0.1% TFA.
HPLC column	C18 Column, 4.6 µm,250 mm
UV wavelength	220 nm
Injection volume	20µl

Flow Rate	1 ml/min
Run Time	30 min

Gradient Programme for DPK 060:

Time (Min)	Pump A	Pump B	Flow Rate (ml/min.)
0.0 – 25.0	25%	75%	1
25.0 – 25.1	50%	50%	1
25.1 – 30.0	100%	0%	1
30.0	Stop		

Preparation of Calibration plot of DPK 060:

Accurately weighed 10.0 mg DPK 060 was transferred to 10 ml olumetric flask and dissolved in Milli Q water, and the volume was made up with Milli Q water (1 mg/ml). This solution was further diluted to make a 100 µg/ml concentration of DPK 060. (Dilution was made with Milli Q water). Subsequently, the calibration plot of the DPK 060 was carried out in the range of 0.5-5 µg/ml.

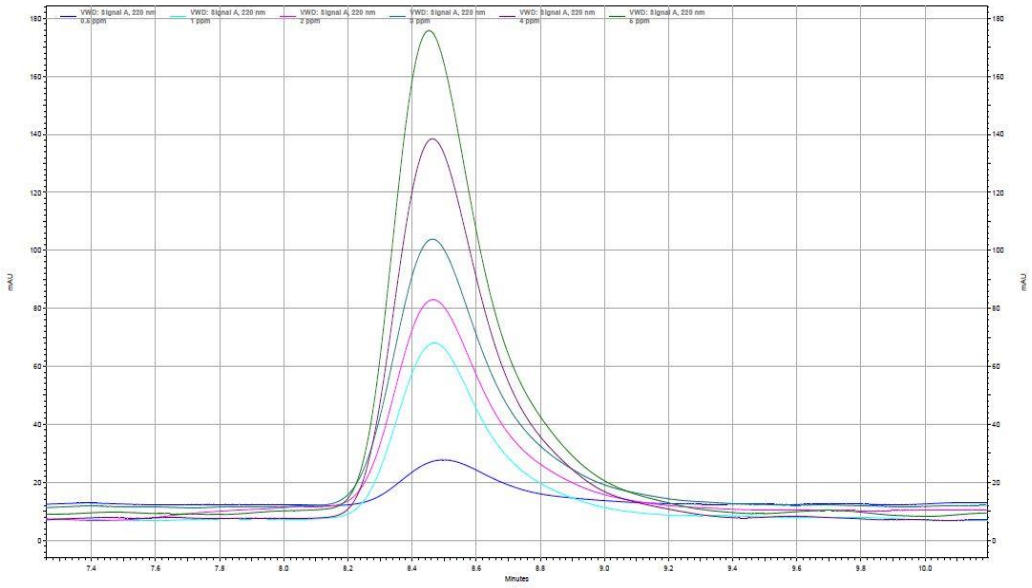

Figure 10a) HPLC overlay chromatogram of DPK 060

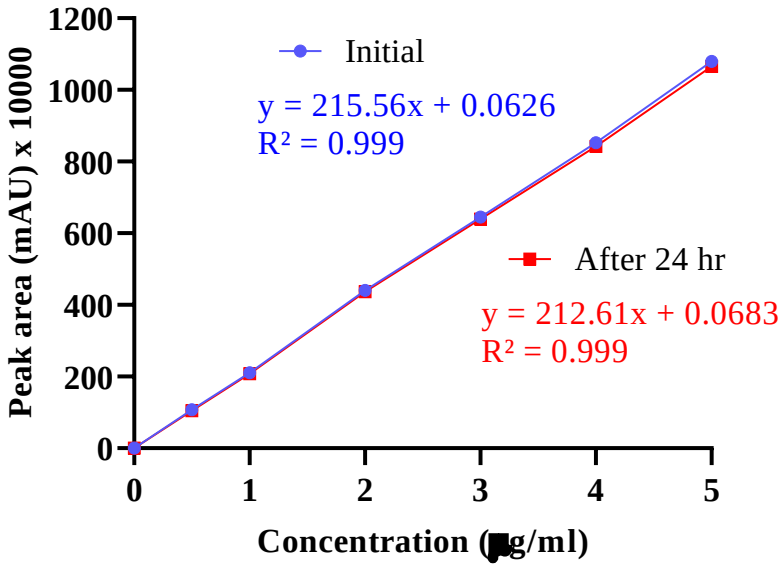

Figure 10b) Calibration plot of DPK-060 by HPLC method

Solid-state analysis (FTIR): FTIR results (figure 11) indicates the excipient compatibility between the DPK 060 and the physical mixture of DPK 060 with excipients.

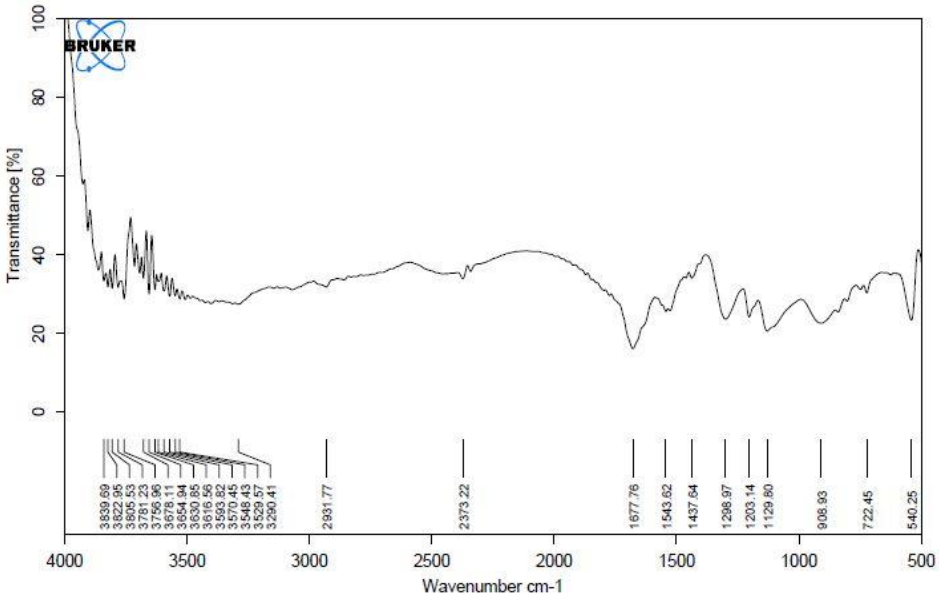

Figure 11 a) FTIR spectra of DPK 060

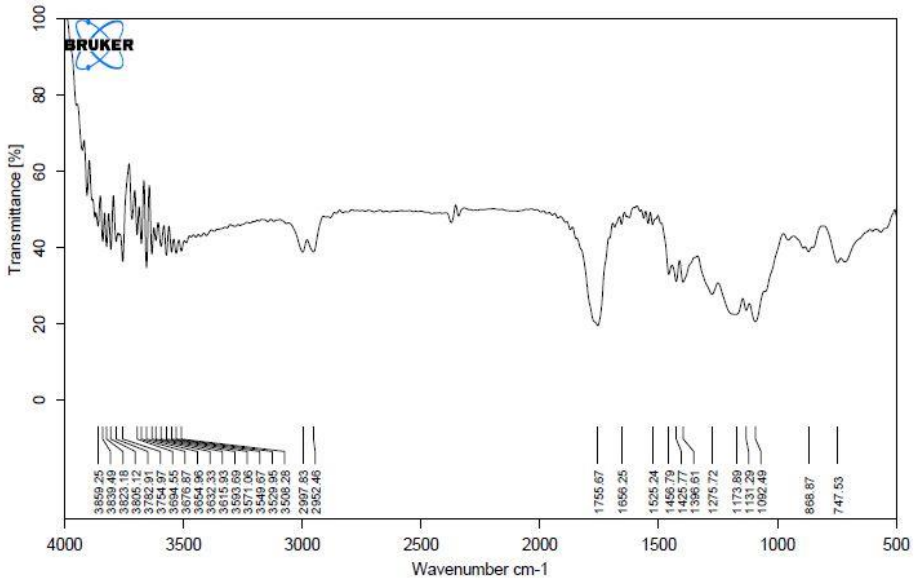

Figure 11 b) FTIR spectra of DPK 060 physical mixture (DPK 60 + Compritol 888ATO + Egg lecithin) (NLC)

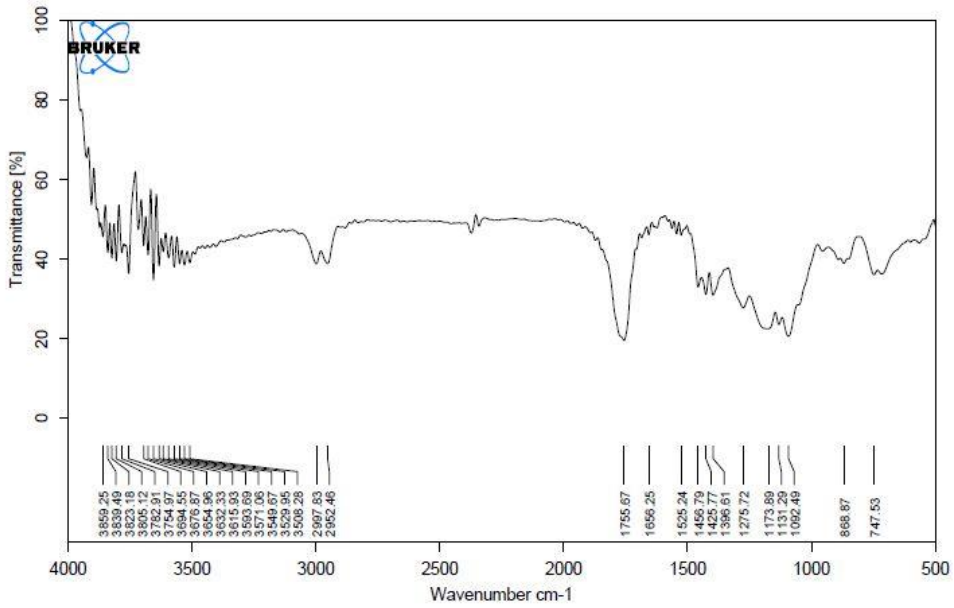

Figure 11 c) FTIR spectra of DPK 060 physical mixture (DPK 60 + Ceteareth-25 + Steareth-10 + PVP K 30) (for lotion)

❖ **DPK 060 Lotion (1%) Formulation:**

❖ Different excipients were screened thoroughly for the preparation of a lotion-based formulation of DPK 060. The optimized batch of the formulation having the following excipients (due to the better compatibility and no interference, were selected for the preparation of the optimized batch).

Optimized Batch: Omiganan (1%) lotion

Ingredients	%Quantity (%w/w)
DPK 060 (1%)	1%
Steareth-10	0.3%
Cetyl alcohol	4%
Ceteareth-25	2.5%
Isopropyl Myristate	5%
Propyl Paraben	0.02%
Propylene Glycol	5%
PVP K30	2%
Methyl Paraben	0.1%
20 mM Acetate buffer pH 5.5	Q.S to 100 %

Method of Preparation (Peptide-based Lotion)

1. The oil phase was prepared by mixing the weighed quantity of cetyl alcohol, steareth-10, ceteareth-25, and isopropyl myristate and heating at 70°C.
2. The aqueous phase was prepared by dissolving weighed quantity of DPK 060 and PVP K 30 sufficient volume of 20 mM acetate buffer pH 5.5.
3. Methylparaben and propylparaben were dissolved in an appropriate volume of 20 mM acetate buffer pH 5.5 and after that propylene glycol was added and this mixture was added into the solution obtained in step 2.
4. The remaining quantity of 20 mM acetate buffer pH 5.5 was added to the aqueous phase and heated at 70°C.
5. The heated Oil Phase was added into the heated aqueous phase and mixed with stirring using a magnetic stirrer. Further, the formed lotion formulation was cooled down to room temperature and filled in the tube. (at the same temperature).

❖ **DPK 060 Nano-lipid constructs (1%):**

DPK 060 loaded nano-lipid constructs (NLCs) were prepared by melt-emulsification – high-pressure homogenization technique. Briefly, solid lipid and liquid lipid were taken in 7:3 ratios (%w/w). The aqueous phase was prepared by dissolving the surfactant in 5 ml of 20 mM acetate buffer pH 5.5. Both these phases were maintained at 75°C and DPK 060 was added into the melted lipid matrix, followed by addition of the aqueous phase to the lipid phase drop by drop under continuous stirring at 700 rpm to get a homogeneous emulsion. The obtained primary emulsion was then subjected to high-pressure homogenization (HPH) for size reduction.

Optimized Batch: DPK 060 Nano-lipid constructs

Parameter	Optimized value
Lipids	Compritol 888 ATO (solid lipid), Miglyol- 840 (liquid lipid), Tween 80, and Egg lecithin (surfactant)
Solid: Liquid Lipid Ratio	7:3 (%w/w)
Stirring speed	700 rpm
HPH parameters	10000 psi, 10 cycles
DPK 060: Lipid	1:4

❖ **Characterization of the DPK 060 Nano-lipid constructs:**

Particle size, zeta potential, and % entrapment efficiency:

- Particle size and zeta potential of the optimized formulation were found to be 128 nm and -22.5 mv respectively and are shown in Figures 12 and 13 respectively. % Drug entrapment of the peptide in the formulation was found to be 85 % with a loading capacity of 6.8%.
- To determine the % drug entrapment, DPK-060 NLC dispersion was centrifuged at 18,000 rpm (4 °C for 30 min). The separated NLC fraction was dissolved in water: methanol (2:8) mixture and analyzed by developed HPLC method. To ensure the mass balance of the drug, the supernatant was also analyzed for the free drug content using the HPLC method at 220 nm. The % drug entrapment and % drug loading were calculated by using the following equations: (32)

$$\%\ \boldsymbol{Entrapment\ efficiency} = \frac{\boldsymbol{Amount\ of\ entrapped\ drug}}{\boldsymbol{Total\ drug\ added}}\ \boldsymbol{x\ 100}$$

$$\%\ \boldsymbol{Drug\ loading} = \frac{\boldsymbol{Drug\ loaded\ (mg)}}{\boldsymbol{Total\ weight\ of\ NLCs\ (mg)}}\ \boldsymbol{x\ 100}$$

Figure 12 Particle size of the developed DPK 060 loaded NLCs

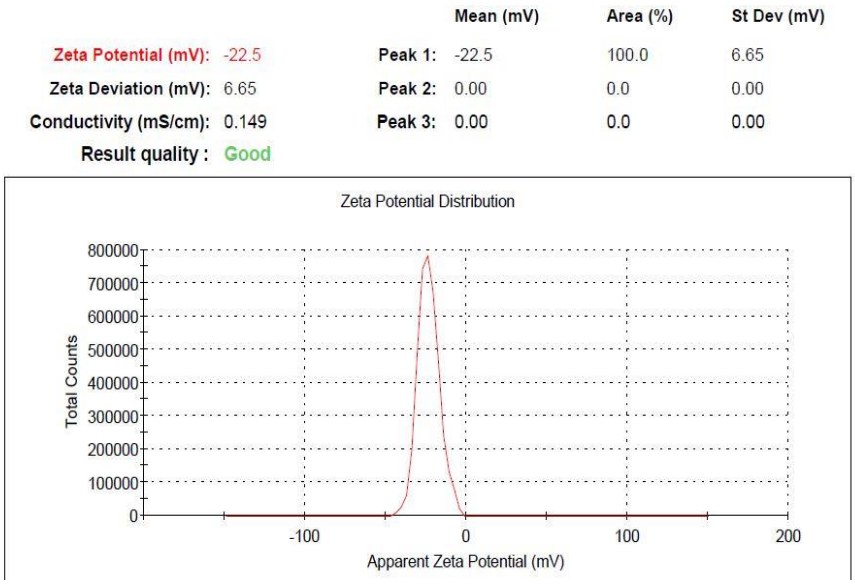

Figure 13 Zeta potential of the developed DPK 060 loaded NLCs

❖ **Scanning Electron Microscopy (SEM):**

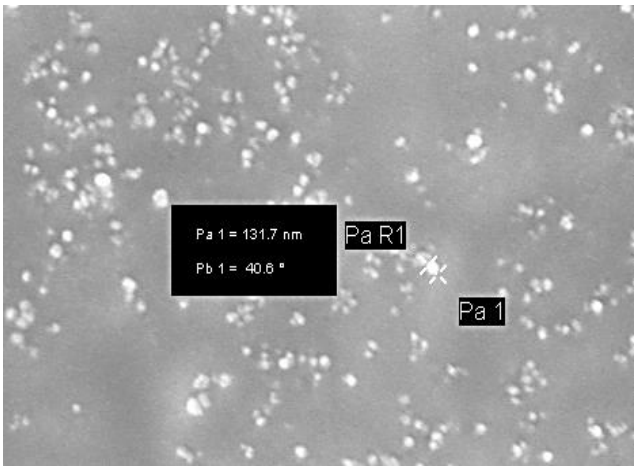

Figure 14 SEM image of the developed DPK 060 loaded NLCs

❖ **Carbopol based gel was formulated as a vehicle for the delivery of DPK 060 loaded NLCs.**

- The gel base formulation was developed and loaded with DPK-060 (1%w/v) and optimized DPK-060 nano-lipid constructs (1%w/v) to improve the viscosity of the formulation in order to enhance the retention on the skin surface. Carbopol 934P polyacrylic acid-based gelling agent is extensively used in topical formulations. Due to the better hydration and wetting properties, Carbopol 934P requires very less hydration time and also forms uniform dispersion. Briefly, weighed quantity of Carbopol 934P (1.2%w/v) was mixed and dispersed in DPK-060 NLC dispersion (1%w/v) using an overhead stirrer at 2000 rpm for 1 h. After complete hydration, the mixture of preservatives namely methylparaben (0.2%w/v) and propylparaben (0.02%w/v) in propylene glycol (4%w/v) was added with continuous stirring, and the pH was adjusted to ~6.5 by dropwise addition of 10% v/v Sodium hydroxide solution. The detailed formulation composition is shown in the following table.

Formulation components	Concentration
DPK 060 NLCs	1% w/v
Carbopol 934P	1.20% w/v
Propylene glycol	4% w/v
Methyl paraben, propyl paraben	0.2 and 0.02 %w/v
Sodium hydroxide solution (10%)	…qs… (pH adjustment of 6.5)

❖ **Characterization of DPK 060 gel:**

❖ **Viscosity of DPK-060 gel**

The viscosity of free DPK-060 gel and DPK-060 NLC gel was measured using cone and plate rheometer (Bohlin C-VOR, Malvern Instruments Ltd., Worcestershire, UK) at 25±1ºC. Briefly, 200 mg of the gel sample was placed on the sample holder and the spindle (plate with 20 mm diameter and 4º cone angle) was lowered and allowed to equilibrate for 5 min. The spindle was rotated at a shear rate of 10/s and corresponding viscosity (Pa.S) was reported (33).

Results: The viscosity of free DPK-060 gel and DPK-060 NLC gel was found to be 13.10 ± 0.292 Pa.S and 16.22 ± 0.451 Pa.S respectively.

❖ **Spreadability of DPK-060 gel**

The spreadability of free DPK-060 gel and DPK-060 NLC gel was evaluated by the reported method (34). Briefly, 500 mg of gel sample was positioned within the circle of 1 cm diameter pre-marked glass plate over which second glass plate was placed. The 500 g weight was applied on the upper glass plate for 5 min and an increase in diameter was noted.

Results: The spreadability of free DPK-060 gel and DPK-060 NLC gel was found to be 6.87 ± 2.07 cm2 and 7.69 ± 1.38 cm^2 respectively. The spreadability of free DPK-060 gel was lesser than DPK-060 NLC gel which may be attributed to the increased solid content of the gel after the addition of NLC formulation.

❖ **pH of DPK-060 gel**

The pH of free DPK-060 gel and DPK-060 NLC gel was determined using a digital pH meter (Lab India Pvt. Ltd, Mumbai). A pH measurement of the samples was performed in triplicate, and the average value was reported.

Results: The pH of free DPK-060 gel and DPK-060 NLC gel was found to be 6.4 ± 0.4 and 6.5 ± 0.4 respectively.

❖ **In-vitro release studies:**

In vitro release of DPK 060 from lotion and NLCs was carried out using a widely used dialysis bag technique. Dialysis bag (Mole. weight - 12 KDa cut-off) containing the free DPK 060 gel, DPK 060 lotion, and DPK 060 NLC gel were placed in a 30 mL of release media (PBS pH 7.4:

methanol in 8:2 ratio). Samples (0.5 ml) were withdrawn at regular time intervals (0.5,1,2,3,4,5,6 and 12 h) from the receptor compartment and the same volume (0.5 ml) was replaced by a fresh diffusion medium. The samples were analyzed using the developed HPLC method. All the experiments were performed in triplicate and the average values were taken.

Results: Results demonstrated that around 84% of DPK 060 was released from the lotion and free DPK 060 gel within 1 h whereas around 72% of DPK 060 was released from NLCs after 6 hr, indicating the sustained release of DPK 060.

❖ **Ex-vivo permeation and skin retention studies:**

Ex-vivo permeation was carried out 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 Franz diffusion cell with a diameter of 9 mm, and free DPK 060 gel (1%), DPK 060 lotion (1%), DPK 060 NLC gel (1%) was added to the donor compartment. The assay conditions were the same as the in the vitro release section. After 12 h, the skin samples were removed from the diffusion cell, and the surface was thoroughly washed with PBS pH 7.4 and cut into small pieces, and then added to a tube containing 5.0 mL of Methanol for homogenization. All samples were vortexed for 1 min and the DPK 060 content was quantified by HPLC.

Results: Results demonstrated that around 89% of DPK 060 was retained on the skin from the lotion and free DPK 060 gel whereas around 68% of DPK 060 was retained on the skin NLCs gel.

❖ **In-vitro MTT assay:**

A study was performed in the 3T3-fibroblast cells to assess the cytotoxicity of the developed formulations. Cells were seeded with 5×10^3 cells/well cell density in a 96 well microtiter plate using DMEM supplemented with 10% Fetal Bovine Serum. The cell culture was grown for 24 hrs in a CO_2 incubator maintained at 5%concentration and humidified with saturated Copper sulfate solution. After 24 hrs, the cells were exposed at different w/w ratio and evaluated for 6 h. After the exposure time, cell media was replaced with a complete medium containing 1% solution of antibiotic and 10% Fetal Bovine Serum. After 24 h, cells were washed with PBS

pH 7.4, and MTT dye (5mg/mL) solution (20 µl) was added to each well plate. The MTT dye was permitted to react for 4 h under incubator condition, after that cell medium in each plate was replaced with 100 µl of DMSO (Himedia, Mumbai) and the microtiter plate was shaken gently to dissolve the crystals of formazan. The color of the formazan was determined using a Biorad microtiter plate reader (Biorad, California) at 570 nm. PBS treated Cells were used as control cells. The absorbance values of cells treated with PBS were taken as 100% cell viability and all other treatments were expressed relative to it (35, 36). DPK 060 in the concentration range of 0.01 – 100 µg/ml was evaluated for the MTT assay with Triton X 100 and PBS pH 7.4 as positive and negative control respectively.

Results: The viability of cells treated with free DPK 060, 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 DPK 060 NLCs have no potential toxic effects on the 3T3-fibroblast cells and are found to be safe for dermal delivery.

❖ **Cellular Uptake by Confocal Microscopy:**

The in vitro cell-based assays were carried out on 3T3-fibroblast cells. Cells were seeded with 1×10^5 cells/well cell density in a 6 well plate using DMEM supplemented with 10% Fetal Bovine Serum. The cell culture was grown for 24 hrs in a CO_2 incubator maintained at 5% concentration and humidified with saturated Copper sulfate solution. To evaluate the cellular uptake, FITC tagged DPK 060 was loaded in the formulations. Briefly, cells were seeded at a cell density of 1×10^5 cells/well in 6-well cell culture plates, allowed to adhere for 24 h followed by treatment with FITC tagged DPK 060 formulations. The cellular uptake was determined at 1 and 4 h treatment by confocal microscopy.

Results: The cellular uptake of the different DPK 060 formulations were depicted in figure 15. Results demonstrated the higher cellular uptake of the DPK 060 loaded NLCs in comparison to free DPK 060 gel and DPK 060 lotion after 4 h, which results in the enhanced therapeutic efficacy of the developed formulation.

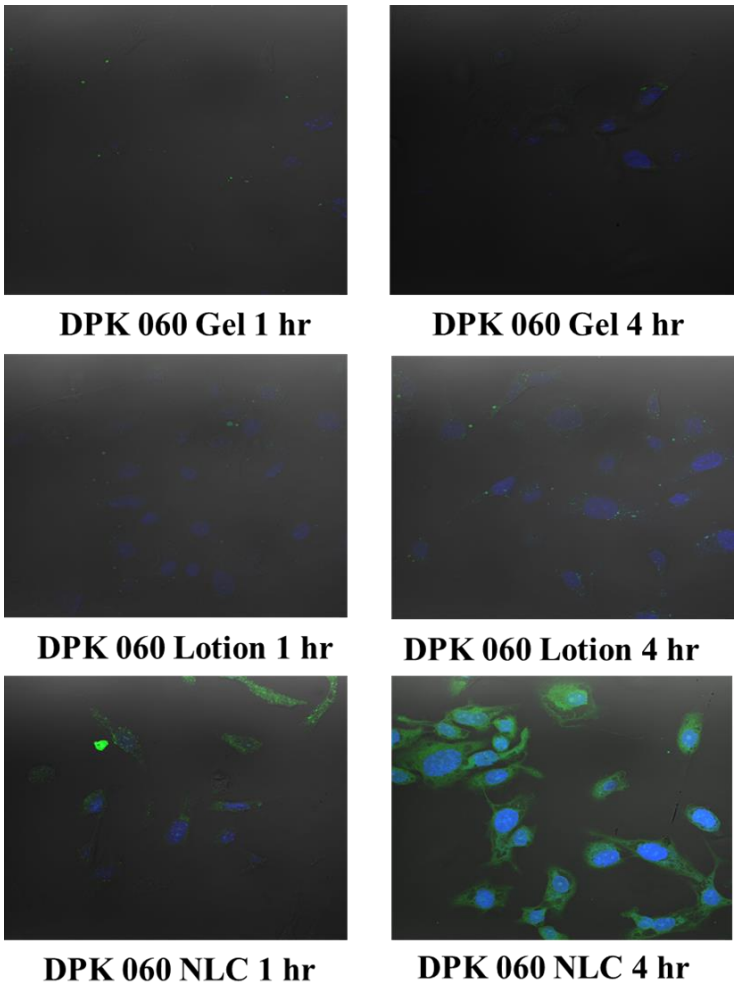

Figure 15 Cellular uptake of DPK 060 formulations

❖ **In-vitro antimicrobial studies:**

The minimum inhibitory concentration (MIC) of developed formulations of Omiganan and DPK 060 were determined by the broth micro-dilution method (41, 42). Briefly, *S. aureus* and *C. albicans* were seeded in 96 well plates at a density of 5×10^3 CFU per well (1×10^5 CFU mL^{-1}, 50 µL). Various concentrations (from 100 to 0.125 µg mL^{-1}, 50 µL) of developed formulations in Mueller Hinton Broth (MHB) medium was added to each well. In this study, Amikacin and Fluconazole were used as a positive control for *S. aureus* and *C. albicans* respectively. Microbes without any treatment were used as negative controls. All the experiments were performed in triplicate. The bacteria were incubated under standard conditions for 16–24 h. The optical density at 600 nm was measured using a microplate reader.

Results: Omiganan loaded formulations exhibited MIC in the range of 12-16 µg/mL and 32-64 µg/mL against *S. aureus* and *C. albicans* respectively. While 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. The results indicate that both of these peptides have strong antibacterial activity against *S. aureus* while less potent to the strain of *C. albicans*.

❖ **Stability studies:**

Short term stability of all developed formulations i.e., Omiganan liposomal and NLC gel, Omiganan lotion, DPK 060 NLC gel, and DPK lotion) was studied at 30±2 °C/65±5 %RH for 3 months. Vesicle/particle size of loaded nanocarriers and % drug content were considered critical and therefore selected as stability-indicating characteristics for the developed formulations. Samples were withdrawn every month and evaluated for critical stability-indicating parameters using the methods described earlier.

Results: The results of the stability studies indicated a slight increase in vesicle/particle size as well as a slight decrease in % drug content. However, the values observed after 3 months were found within desirable limits required for formulations to perform effectively

Formulation	Time (months)	Vesicle/particle size	Drug content (%)
Omiganan Liposomes	Initial	121.7 ± 2.50	72.52 ± 1.12
	1 month	125.4 ± 2.24	71.99 ± 1.34
	2 month	128.9 ± 3.02	71.25 ± 1.46
	3 month	130.1 ± 2.77	70.68 ± 1.73
Omiganan NLCs	Initial	118.7 ± 2.01	79.82 ± 1.37
	1 month	121.5 ± 2.49	79.15 ± 1.30
	2 month	124.2 ± 3.25	78.89 ± 1.62
	3 month	125.9 ± 3.12	78.41 ± 1.49
Omiganan lotion	Initial	--	99.78 ± 1.58
	1 month		98.91 ± 2.10
	2 month		98.15 ± 1.84
	3 month		97.07 ± 1.93
DPK 060 NLCs	Initial	126.9 ± 2.32	84.68 ± 1.27

	1 month	128.7 ± 2.76	83.96 ± 1.75
	2 month	129.2 ± 3.31	83.24 ± 1.64
	3 month	134.3 ± 3.18	82.88 ± 1.78
DPK lotion	Initial	--	99.91 ± 1.45
	1 month		99.07 ± 1.78
	2 month		98.19 ± 1.44
	3 month		96.53 ± 2.07

❖ **Ongoing Studies:** In-vivo studies

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