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# In-vitro and In-vivo Characterization and Preparation of Silica Nano-formulation Hydrogel for the Treatment of Atopic Dermatitis

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**KEYWORDS**

Silica nanoparticles; Atopic dermatitis; Topical drug delivery; Solubility enhancement; Histopathology study

**Abstract**

Atopic dermatitis (AD) is a repetitive inflammatory skin disorder with limited treatment options. Innovative targeted therapies are gaining significant interest and momentum towards disease control including better ways to deliver drugs topically. Tacrolimus is one such compound which is used to manage moderate to severe AD without causing atrophy which is one of the common side effects of steroids. However, Tacrolimus suffers from poor solubility and retention in the skin when used alone in hydrogel. Therefore, we have prepared Tacrolimus loaded mesoporous silica nanoparticles (TMSNs) to overcome the issues related to its solubility and effective topical delivery. Mesoporous silica nanoparticles (MSNs) were synthesized using sol gel technique and surface functionalized using amino (–NH2+) and phosphonate (-PO3-) groups. Tacrolimus was loaded into MSNs and the particles were characterized for particle size (TEM and DLS), zeta potential (DLS), solubility studies, FTIR, TGA, XRD, BET and cytotoxicity studies. Water solubility of Tacrolimus was increased by 7 folds with phosphonate functionalized MSNs compared to free Tacrolimus. Further the TMSNs were incorporated in to carbopol gel, and the gel formulation was evaluated for various gel characterization tests (pH, spreadability, viscosity), in vitro tests (drug release, permeability studies) and in vivo tests (skin irritation study and efficacy studies) using 1-Fluoro- 2,4-dinitrobenzene (DNFB) induced dermatitis in Balb/c mice. Results of in vitro and in vivo study showed that TMSNs loaded gel showed significantly higher amount of Tacrolimus retained (ex vivo – rat skin) and much higher reduction in ear thickness and improved histology (in vivo - in mice). Our data collectively suggest that MSNs incorporated hydrogel as a promising new formulation strategy for topical delivery of poorly soluble drugs.

## 1. Introduction

Atopic dermatitis (AD) affects around 10 % of adults and 20 % of children across the globe with the clinical features including intense itch, pruritus and recurrent eczematous lesions. Though the occurrence of AD is under control in high income countries, its increasing number in rest of the world demands better therapeutic interventions. AD can start at very early age such as 3–6 months of age, however, evidences suggest that occurrence in both persistent and new onset form is ordinary in adults. AD has complex pathophysiology which involves a strong genetic predisposition, epidermal barrier disruption, and T-cell driven immune dysfunction. [1] There are increasing evidences that AD involves multiple immune pathways like JAK- signal transducer and activator of transcription (STAT) and spleen tyrosine kinase pathways, type-2 inflammatory pathway out of which type-2 inflammatory response is dominant. Currently, “reactive management” strategy is being followed to treat AD which includes antagonistic response to acute flare ups with antiinflammatory agents. However, normal appearing skin may have subclinical keratosis and inflammatory cell infiltration which require antiinflammatory therapies. Topical corticosteroids are the first line therapies for this aggravated disease condition, however, the extended application of which can lead to skin atrophy and other side effects. [2] Therefore, more efficient alternative treatment can be developed which overcome the drawbacks associated with long-term use of corticosteroids. Tacrolimus is an immunosuppressant and belongs to macrolide class. It was isolated from the fungus *Streptomyces tsukubaensis* and shown to possess prominent therapeutic efficacy in inflammatory conditions including AD. It binds to cytoplasmic immunophilins and selectively inhibits phosphatase activity of calcineurin which ultimately inhibits early T-cell activation (i.e. immunosuppression) and prevents gene expression of several proinflammatory

Journal of Chemical Health Risks

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cytokines including interleukin-2 (IL-2) and interferon-γ. However, Tacrolimus is a class II molecule of BCS classification owing to its low solubility (4–12 μg/ml) and high permeability. [3] Additionally, Tacrolimus cannot readily cross the stratum corneum due to its physicochemical properties like high molecular weight (822.95 g/mol) and strong lipophilicity (partition coefficient log P = 3.96 ± 0.83). It was evident in the report that a major portion of the drug remained in the stratum corneum upon topical/dermal application. Furthermore, long term use of Tacrolimus shows many adverse effects like burning sensation with subsequent pain and redness, pruritus at the application site with probability for serious drug interactions. Hence, it is very challenging to incorporate Tacrolimus into the formulation and deal with the issue of varied bioavailability. In 1999, US-FDA approved Tacrolimus ointment Protopic for the treatment of moderate to severe AD and has shown to be effective in children with minimal systemic absorption. [4] The ointment, however, presents its own disadvantages like difficultly in washing them off from the skin surface and leaves a sticky sensation behind due to their greasy nature. A recent report demonstrated that response to the Tacrolimus ointment is higher when applied under occlusion or to the face and intertriginous areas where the skin is thinner and more permeable. This provokes the need to develop a new delivery system which targets the Tacrolimus molecule to the deeper layers of the skin while avoiding systemic delivery. It is also expected that the new delivery system should allow greater penetration without producing adverse effects. Nanoparticle-based formulations are one of the best approaches for drug delivery to improve bioavailability of BCS class II drugs such as Tacrolimus. [5] However, they are mostly explored for systemic/oral delivery. Erdogan *et al.* developed liposomal lotion of Tacrolimus for topical application and showed higher dermal concentration as compared to the IV injection. Uno *et al.* developed water-in-oil-in-water (W/O/ W) type multiple emulsion of Tacrolimus and provided better immunosuppressive effects in the targeted site without being absorbed systematically. Later on, a lipid nanoparticles loaded with Tacrolimus was developed by Pople *et al.* for topical application in the treatment of AD which showed good entrapment efficiency (96.6 %) and stability. However, lipid based nanocarriers possess disadvantages like lipid degradation, drug leaching and lack of controlled release of the drug. [6] These unmet needs give rise to the development of more suitable nanoformulation in the treatment of AD. The application of ordered mesoporous materials in drug delivery for the first time. High drug loading and controlled release of the drugs can be achieved because of unique properties of MSNs such as ordered structure, high surface area, tuneable pore volume and size, biocompatibility and very good stability. In addition, surface of the MSNs can be chemically functionalised through grafting of different organic functional groups to the free silanol groups by covalent interactions. [7] Use of different types of silica nanoparticles have been reported for topical drug delivery, however their use in the treatment of AD is not explored to a great extent. In the present study, we have reported the development of MCM-48 type of mesoporous silica nanoparticles loaded with Tacrolimus which provided high loading capacity, improved solubility and local bioavailability enhancement. They have higher skin penetration, enhanced drug accumulation at the target site of AD, i.e. resident immune cells of the dermis and epidermis. Tacrolimus loaded mesoporous silica nanoparticles (TMSNs) were prepared by modified sol–gel technique and characterized for *in vitro* and *in vivo* using DNFB induced dermatitis model in mice. [8]

## 2. Materials and Methods

### 2.1. Materials

Tacrolimus was kindly gifted by Concord Biotech Ltd., India. Cetyltrimethylammonium bromide (CTAB), Poloxamer 407 (F127), Tetraethyl orthosilicate (TEOS), (3-Aminopropyl) triethoxysilane (APTES) and 3-(trihydroxysilyl) propyl methylphosphonate monosodium salt solution 50 wt% in H2O (THMP) was procured from Sigma Aldrich, India. Carbopol 934 was obtained from Sigma Aldrich, India. HPLC grade water and Acetonitrile were purchased from Merck Ltd., India. IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Abcam, India.

### 2.2. Preparation of MCM-48

A reported method for the synthesis of re-dispersible MSNs by Kim *et al.* was modified and used for the synthesis of MSNs. First CTAB: F127 in 1:2 ratio was dissolved in the mixture of 100 % ethanol (85 ml) and 2.9 wt% aqueous solution of ammonia (213 ml) followed by the addition of TEOS (3.86 ml) under continuous stirring. The mixture was stirred for 1 min at 1000 rpm,

Journal of Chemical Health Risks

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JCHR (2024) 14(4), 406-419 | ISSN:2251-6727

room temperature. Resultant mixture was kept under static condition for 24 h at room temperature. The product was collected by centrifugation at 14,000 rpm for 15 min, washed twice with water and twice with ethanol, and dried overnight in hot air oven at 60 ◦C. Calcination was performed at 550 ◦C for 5 h to remove the surfactant. Calcined MSNs were denoted as MSNs-C. [9]

### 2.3. Functionalization of the prepared particles

**Amino functionalization:**

2 g of uncalcined MSNs were dispersed in 125 ml methanol under continuous stirring at 750 rpm. 7.5 ml APTES was added to the above suspension and left to react for 24 h under stirring at 750 rpm. Centrifugation was carried out at 14,000 rpm for 10 min to collect the amino functionalized MSNs (MSNs-NH2). The collected MSNs-NH2 were, washed twice with methanol to remove the access material and dried in an hot air oven at 60 ◦C overnight. Solvent extraction procedure was used for the removal of surfactant. [10]

**Phosphonate functionalization:**

THMP was dissolved in water to obtain the concentration of 1 % v/v. Initially the pH of the solution was around 12 which was adjusted to 5–6 using 0.1 N HCL. This acidification is required to catalyse the process of silanol group condensation on silica surface and to prevent hydroxylation of silica and its dissolution during the grafting process. To this solution 100 mg uncalcined MSNs were added and the overnight reflux was carried out 100 ◦C. The phosphonate functionalized MSNs (MSNsPO3) were collected by centrifugation (14,000 rpm, 10 min), washed twice with fresh water and twice with ethanol, and dried in an hot air oven at 60 ◦C overnight. Solvent extraction procedure was used for the removal of surfactant. Solvent extraction was performed by dispersing MSNs-NH2 / MSNs PO3 (150 mg) in a mixture of 20 ml methanol and 1 ml 32 % w/w aqueous HCl. The suspension was kept under stirring at 1000 rpm and 60 ◦C for 24 h. The particles were collected by centrifuging at 14,000 rpm followed by washing twice with fresh water and twice with ethanol. Collected MSNs-NH2 were then dried in an hot air oven at 60 ◦C overnight. [11]

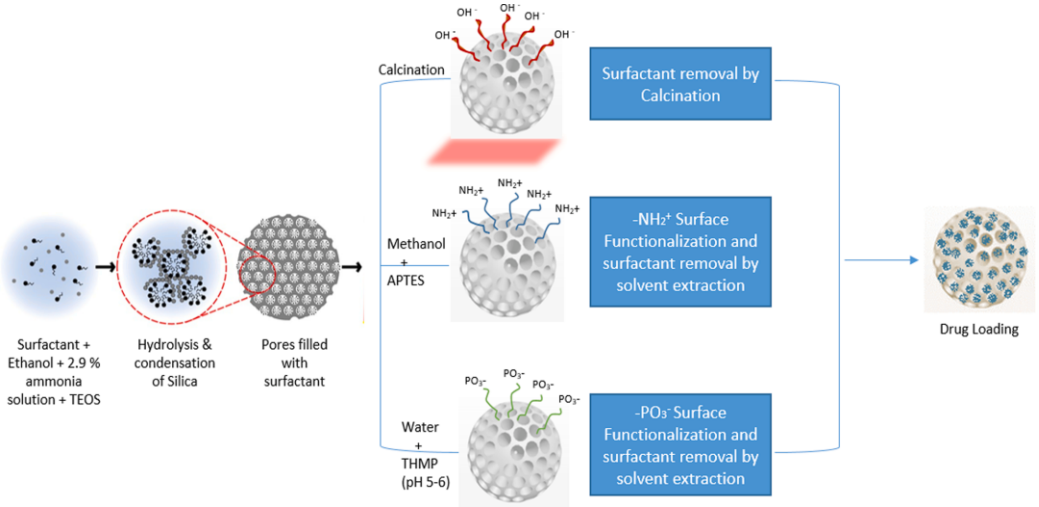


**Fig. 1.** Schematic diagram of synthesis process, surface functionalization and Tacrolimus loading in MCM-48 type of MSNs.

### 2.4. Drug loading

Drug loading was performed using previously established protocol in our laboratory. MSNs were taken (80 mg, 90 mg, and 95 mg for 20%, 10% and 5% drug loading respectively) and dispersed in 2 ml of ethanol. Sufficient quantity of the Tacrolimus was taken to make total solid content 100 mg and was dissolved in another 1 ml of ethanol. Drug solution was added to the MSNs suspension and the resultant suspension was sonicated in bath sonicator for 1 h. Then the drug loaded MSNs were collected by removing the solvent using a rotary evaporator. This procedure was done for all the 3 types of MSNs followed by the solubility studies. The particles with highest solubility were further selected for other experiments. Drug loaded functionalized MSNs were denoted as TMSNs-C, TMSNs-PO3 and TMSNs-NH2

Journal of Chemical Health Risks

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for Calcined, Phosphonate functionalized and Amino functionalized MSNs respectively. [12]

**2.5. MSNs characterization**

**Particle size & Zeta potential:**

The hydrodynamic diameter of the MSNs was measured by dynamic light scattering (DLS) using Zetasizer, Nano SZ, Malvern. Detection angle was set at 173◦, system temperature at 25 ◦C and equilibration time set to 60 sec. The samples were dispersed in water (0.1 % w/v), sonicated for about 20 min and diluted as required before measurement. Zeta potential measurements of MSNs were performed using the same instrument. Undiluted samples were used for zeta potential measurement. Results were recorded in triplicate and expressed as the mean ± SD of three runs.

**TEM:**

The MSNs were dispersed in ethanol and sonicated for 30 min. Carbon-coated copper grids were dipped in the suspension and the deposited suspension (~5 µl) was allowed to dry for at least 24 h. Transmission electron microscopy (TEM) images were obtained with a Hitachi 7700 microscope operated at 80 kV. Different images were taken over different magnification.

**TGA:**

A thermo gravimetric analysis (TGA) was performed using TGA/ DSC 2 STAR System, Mettler Toledo. Heating protocol was set to heat the samples at a rate of 10 ◦C per min from 50 ◦C to 900 ◦C in an air flow. Samples were equilibrated at 50 ◦C, before initiating the measurements. Total weight loss was calculated from the respective thermogram.

**FTIR:**

Fourier transform infrared spectra (FT-IR) were recorded by Spectrum Two Spectrometer, PerkinElmer with LiTaO3 (lithium tantalate) MIR detector. The equipment was designed for real time studies and results were recorded in vacuum and controlled atmosphere. The sample cell of was equipped with KBr windows and the samples suitable for transmission of IR rays were loaded. The equipment was set to work at the resolution of 16 cm- 1 over 30 scans.

**Solubility studies:**

Solubility studies were performed in distilled water and PBS containing 1% w/v Brij O20 (VPBS) using rotating test tube mixer. 5 mg of pure drug and drug loaded MSNs equivalent to 5 mg drug were added in 2 ml of water / VPBS and mixed using a vortex mixer. The centrifuge tubes were kept on the rotating mixer for 24 h. After 24 h, the suspensions were subjected to centrifugation (Centrifuge 5424, Eppendorf) at 14,000 rpm for 10 min and supernatant was filtered through 0.22 µm syringe filters. The filtrate was analysed at 213 nm in a UHPLC system (Agilent Technologies 1290 Infinity; Cosmosil C18 column, 150 mm × 4.6 mm; acetonitrile:water in the ratio of 70:30 as mobile phase; flow rate at 1.0 ml/min with injection volume of 20 µl; R2: 0.999) to estimate the dissolved drug content. All the experiment was performed in triplicate.

**Nitrogen (N2) adsorption–desorption analysis:**

Total surface area, pore size and pore volume of blank and drug loaded MSNs were measured by N2 adsorption–desorption isotherms at-196 ◦C using Tristar II, Surface area and Porosity analyser (Micromeritics). Prior to analysis, samples were degassed at 80 ◦C using VacPrep 061, sample degas system (Micromeritics) overnight. BrunauerEmmet-Teller (BET) technique was used to estimate the total surface area. The average pore size and volume were estimated from the adsorption branch of the isotherms as per the method proposed by Barrett-Joyner-Halenda (BJH), Kruk-Jaroniec-Sayari equations.

**2.6. Cytotoxicity assay**

To determine the cell viability of TMSNs-PO3, the alamar Blue assay was performed as per the manufacturer's protocol. Briefly, HaCaT cells were seeded in a 96 well plate at 10,000 cells per well in 100 µl of DMEM (10 % FBS). After overnight incubation at 37 ◦C and 5 % CO2, cells were treated with TMSNs-PO3 and pure drug suspension for 24 h and medium (-ve control). Each sample was tested in triplicate. The cell culture medium was replaced with 10 µl alamar Blue reagent in an amount equal to 10 % volume in the well. The absorbance was recorded at a wavelength of 570 and 600 nm. % reduction of alamar Blue reagent were calculated with following formula:

% Reduction of alamar Blue = [(Eoxi600 × A570)-(Eoxi570 × A600)/ (Ered570 × C600) - (Ered600 × C570)] × 100

Where,

Journal of Chemical Health Risks

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JCHR (2024) 14(4), 406-419 | ISSN:2251-6727

Eoxi570 = molar extinction coefficient (E) of oxidized alamar Blue at 570 nm = 80586

Eoxi600 = E of oxidized alamar Blue at 600 nm = 117216 A570 = absorbance of test wells at 570 nm

A600 = absorbance of test wells at 600 nm

Ered570 = E of reduced alamar Blue at 570 nm = 155677 Ered600 = E of oxidized alamar Blue at 600 nm = 14652 C570 = absorbance of negative control well (media, alamar Blue, no cells) at 570 nm

C600 = absorbance of negative control well (media, alamar Blue, no cells) at 600 nm

**2.7. Expression of pro-inflammatory cytokines IL-6 in HaCaT cells**

IL-6 is a critical cytokine involved in diverse immunological diseases including atopic dermatitis. Most importantly, this cytokine is involved in CD4 + Th17 immune cell differentiation and maintenance which promoted production of IL-17 and IL-22. IL-6 was also reported to promote CD4 + T cells differentiation to Th2 phenotype through nuclear factor of activated T cells and produce Th2 cytokines including IL-4 and IL-13. Moreover, Th2 cytokines were also reported to increase production of IL-6 from keratinocytes maintaining the inflammatory cycle in the skin. These showed that IL-6 plays an important role in dermatitis initiating both Th2 and Th17 immune responses and is one of the best markers representing skin inflammation in keratinocytes. Therefore, the HaCaT cells were seeded at the density of 150,000 cells per well in 24 well plates. After 16 h of incubation, cells were pre-treated with TMSNsPO3 having 1 ng/ml Tacrolimus in it for 2 h and then stimulated with 1 µg/mL LPS. The supernatants were collected at 24 h and the level of IL-6 was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions. [13]

**2.8. Formulation of MSNs enriched gel**

The TMSNs-PO3 were incorporated into carbopol gel to obtain final concentration of the drug 0.1 % w/w. TMSNs-PO3 were added to carbopol 934 gel base (1.25 % w/v) previously prepared by slowly adding carbopol powder into water under constant stirring and allowing complete swelling of the carbopol overnight. The prepared gel base was neutralized with 0.05 % triethanolamine (TEA). Separately a gel containing 0.1 % w/w pure Tacrolimus was also prepared which was used for comparison. The prepared gel with and without silica was evaluated for viscosity, spreadability and pH.

**2.9. Characterization of gel**

**Rheological measurements:**

a) Viscosity: Viscosity was measure using Discovery HR-3 hybrid rheometer. The temperature was increased in ascending order from 8 ◦C to 40 ◦C with constant shear stress of 10 Pa and soaking time of 120 sec. The gap between platform and spindle was 200 µm and velocity of the spindle was set at 10 rad/sec. The samples were equilibrated prior to the measurements.

b) Spreadability: The measurement of TMSNs-PO3 gel spreadability was done on a wooden block with a glass slide apparatus. Weighed amount of TMSNs-PO3 gel was uniformly applied between the upper and lower plate of the apparatus. Then a weight of 100 g was added to the pan attached to the upper plate via pulley. The time taken by the upper plate to traverse across the length of the lower plate was recorded.

c) pH : pH was measured using digital pH meter with glass electrode. Electrode was dipped in an undiluted gel formulation and readings were recorded. [14]

**2.10. In vitro drug release study**

*In-vitro* release studies were carried out on a 20 ml Franz-diffusion cell which had a diffusion area of 2.92 cm2. The temperature of cell was regulated at 32 ◦C ± 0.5 ◦C with the help of a circulating water in the jacket of the cell and the Franz-box. Dialysis membrane was used for the *in vitro* drug release from 0.1 % w/w Tacrolimus gel and 0.1% w/w TMSNs-PO3 gel. Tacrolimus is practically insoluble in water and buffer solutions; hence, sink condition in the receptor compartment was maintained by adding 1 % w/v Brij O20 to pH 7.4 phosphate buffer solution (PBS) and filled in the receptor compartment. An equivalent amount of the prepared TMSNs-PO3 gel and Tacrolimus gel (reference product) with 0.1 mg of the drug were applied over the dialysis membrane and spread evenly to cover the diffusion area. The donor compartment was covered with Parafilm to prevent water loss. Sampling was done at fixed times up to 24 h and receptor media was replaced with equal volume of fresh media. The Tacrolimus concentration in the collected samples was analysed by HPLC, 1260 infinity, Agilent Technologies. In brief, Nucleosil C18

Journal of Chemical Health Risks

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JCHR (2024) 14(4), 406-419 | ISSN:2251-6727

column was used. Mobile phase consisted of acetonitrile:water in the ratio of 70:30. Column temperature was set to 60 ◦C, flow rate at 1.0 ml/min with injection volume of 50 µl and the detection was done at wavelength of 213 nm (R2: 0.997). The experiment was carried out in triplicate. [15]

### 2.11. Ex vivo skin permeation and skin retention study

The full thick skin of adult Wistar albino rats were used for this study. The skins were shaved and excised ethically and hydrated with PBS. Then the sections were mounted on Franz-diffusion cell with stratum corneum remaining in the contact with the donor compartment and the dorsal skin completely in contact with the receptor compartment. An equivalent amount of the prepared 0.1 % w/w TMSNs-PO3 gel and 0.1 % w/w Tacrolimus gel with 0.1 mg of the drug were applied over the mounted skin and spread evenly to cover the diffusion area. The donor compartment was covered with Parafilm to prevent water loss. The receptor compartment was filled with pH 7.4 PBS with 1 % w/v Brij O20. Permeation across the excised skin was evaluated over a time period of 24 h. The left over formulations were removed using cotton swab and the stratum corneum was separated using tape stripping method. The skin was washed with fresh PBS and divided into small pieces. All the pieces were placed in a 15 ml conical tube containing 5 ml of the mobile phase; homogenized and sonicated to extract the Tacrolimus. The tapes were also collected in a beaker containing mobile phase and sonicated for 1 h to extract the drug. Subsequently, the solutions were filtered through a 0.45 µm syringe filter. The amount of Tacrolimus deposited within the skin was quantified using HPLC at 213 nm. [16]

### 2.12. In-vivo studies

**Skin irritation study:**

*In-vivo* animal studies and the protocols for the same were approved by the Institutional Animal Ethics Committee (Approval No: IP/PCEU/ PHD/26/2019/025). Wistar albino rats (200–300 g) were used to evaluate irritation potential of prepared TMSNs-PO3 gel. Animals were divided in to 4 groups containing 3 rats each. 1 % v/v formalin solution was used as positive control and 1.25 % w/v carbopol gel base acted as a negative control in the study. In the remaining 2 groups, one group received 0.1 % w/w TMSNs-PO3 gel and the other groups received 0.1 % w/w Tacrolimus gel. The formulations containing 0.1 mg equivalent amount of Tacrolimus was applied to their respective groups over the shaved skin. The erythema and edema scores were noted depending on the severity of the skin reaction over the time period of 24 h. The grading of the skin reaction was done as per the testing guideline for acute dermal irritation/corrosion i.e. OECD Guideline 404. Briefly, the standards were kept as 0 for no reaction, 1 for Slight reaction with barely perceptible light pink, 2 for Moderate reaction with dark pink, 3 for Moderate to severe reaction with light red and 4 for severe reaction with extreme redness. *In vivo* efficacy study in BALB/c mice with AD like skin lesions All animal experiments were conducted as per institutional ethics approval (Approval No: IP/PCEU/PHD/26/2019/025). Disease was induced in adult BALB/c mice of mix gender (n = 6 in each group). Following a week of acclimatization, hapten induction method was used to induce AD on the ears of mice (Jin et al., 2009). Skin was sensitized initially by topical application of 0.5 % w/v dinitro fluorobenzene (DNFB) solution prepared in vehicle of acetone:olive oil in the ratio of 80:20. Animals were challenged with application of 20 µl, 0.2 % w/v DNFB solution on both the sides of the ears, after 5 days of sensitization to induce inflammation. This was repeated every alternate day for 2 weeks to induce Th2 response and develop severe disease phenotype mimicking human AD. The treatment was started on day 21 of the disease induction and animals were treated with either 0.1 % w/w TMSNs-PO3 gel or 0.1 % w/w Tacrolimus gel (100 mg) for 7 days (till the animals cured completely). Response to the treatments for AD was observed by grading skin reactions and measuring ear thickness using a digital micrometer. The efficacy was assessed by calculating improvement index of both the formulations with respect to the placebo. These results were also confirmed histologically via hematoxylin and eosin (H&E) staining. [17]

## 3. Results and Discussion

### 3.1. Characterization of MSNs

Selection of ingredients play important role in the synthesis of MSNs as particle size and pore size are dependent on the ratio of different ingredients. A reported method by Meryem *et al* was used to synthesize colloidal mesostructured silica nanoparticles. CTAB: F127 were used in different ratios (1:8, 1:4, 1:2) and

Journal of Chemical Health Risks

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JCHR (2024) 14(4), 406-419 | ISSN:2251-6727

particles were obtained in the range of 90 nm to 300 nm. Decrease in the amount of F127 leads to increase in the particle size. Further decrease in the amount of F127 gave larger aggregated particles in the range of 450–500 nm. On the contrary, further increase in the amount of F127 (>1:8) produced polydisperse particles as reported in the literature. [18] In the current study we intended to obtain particles in the range of 200–300 nm to prevent the drug from entering the systemic circulation and to achieve maximum drug penetration and retention in the skin. Hence, out of all the prepared batches MSNs with average particle size of 280.2 ± 4.84 nm were selected for further studies. Functionalization of the prepared particles is usually done to improve their physical and chemical properties. Surface properties like host–guest binding, hydrophilicity-hydrophobicity, modification of mechanical or optical properties or alteration of surface reactivity can be tuned by surface functionalization of the MSNs. Hence, to improve the loading and the solubility of the Tacrolimus, the surface of MSNs was functionalized with amino and phosphonate groups using APTES and THMP respectively. For functionalization, post synthesis grafting strategy was used. The drug loading was performed in all the three types of particles i.e. MSNs-C, MSNs-NH2 and MSNs-PO3. The particle size was measured using DLS and TEM. [19]

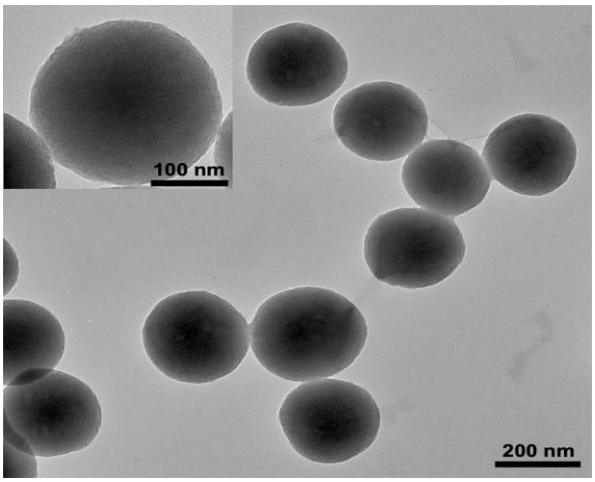


**Fig. 2.** TEM images of synthesized MSNs after calcination. Inset is high resolution image showing porous structure of synthesised MSNs-C.

After calcination particles are relatively spherical with size of ~ 200 nm. After tacrolimus loading, the morphology of both MSNs-NH2 and MSNs-PO3 remains relatively similar with no significant changes compared to calcined particles. Mean particle size of uncalcined MSNs, MSNs-C, MSNs-NH2 and MSNsPO3 and Tacrolimus loaded particles viz. TMSNs-C, TMSNs-PO3 and TMSNs-NH2 were obtained as mentioned in. Particle size of TMSNs-PO3 was increased after drug loading while for TMSNs-C and TMSNs-NH2 it decreased slightly. This increase in particle size could be because of increased hydrophobicity imparted by Tacrolimus especially for TMSNs-PO3 and therefore particles are difficult to redisperse, and larger agglomerates may lead to increased size. The TEM images show that the particle size is ~ 220 nm which much smaller compared to Zaverage measured by DLS. The size of particles measured by DLS was larger compared to TEM, which could be due to the presence of a hydration layer surrounding the particles and possible aggregation in solution. [20] All the particles have narrow poly dispersity index (PDI) < 0.35 which indicates particles are uniform and well dispersed. The zeta potential value for uncalcined MSNs was 14.96 ± 0.11 mV and for MSNs-C it was - 31.52 ± 1.15 mV which confirms successful removal of surfactant upon calcination which imparts positive charge on the surface. The functionalization of MSN was confirmed by zeta potential variation observed in respective functionalized groups. The zeta potential value increased to 35.9 ± 1.65 for MSNs-NH2, while it decreased to - 43.26 ± 1.59 mV for MSNs-PO3 after functionalization with amino and phosphonate groups. After loading Tacrolimus into the particles, the zeta potential of MSNs-C changed from - 31.52 ± 1.15 mV to –23.33 ± 0.60 mV, while in case of MSNs-NH2 it decreased from + 35.9 ± 1.65 to + 12.9 ± 2.45 mV and for MSNs-PO3 it decreased from - 43.26 ± 1.59 mV to almost neutral - 2.9 ± 2.74 mV indicating the

Journal of Chemical Health Risks

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shielding of surface charge due to successful encapsulation. This could be attributed to the deposition of drug on the surface of MSNs. [21]

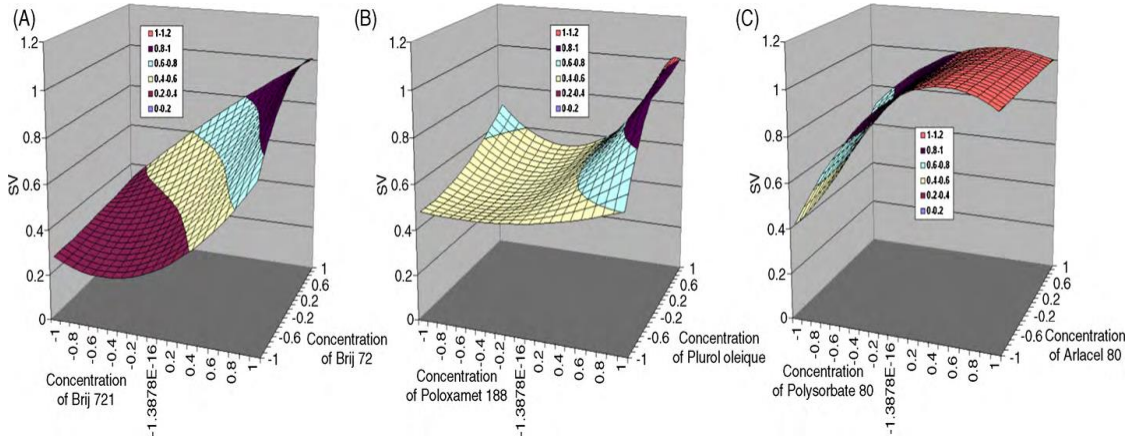


**Fig. 3.** Response surface plots for optimization of surfactant concentration; Brij 721–Brij 72 (A), poloxamer 188–plurol oleique (B) and polysorbate 80–sorbitan monooleate (C) as hydrophilic and hydrophobic surfactant combination.

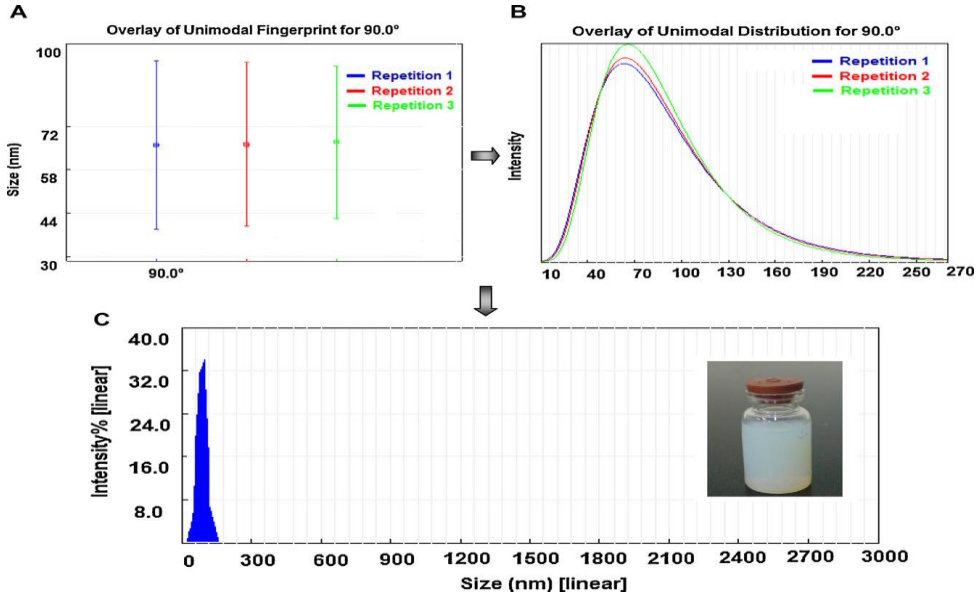


**Fig. 4.** Particle size analysis of T-LN. (A) Unimodal fingerprint and (B) Unimodal distribution for three repetitions of T-LN; (C) SDP histogram of particle size profile obtained by photon correlation spectroscopy (T-LN dispersion photograph in inset).

Further the TGA was performed to confirm the functional group grafting and drug loading into MSNs. The functionalization and drug loading was confirmed from the obtained thermograms and the percentage loading of Tacrolimus into MSNs by weight was also calculated. Thermograms in revealed primary weight loss close to 100 ◦C probably because of adsorbed moisture which results in gradual fall across the complete temperature range due to surface dihydroxylation. Along with removal of water, MSNs-NH2 showed major weight loss close to 370 ◦C and MSNs-PO3 at 540 ◦C as the decomposition of the aminopropyl and phosphonate chains respectively. In drug loaded formulations, TMSNsC, TMSNs-NH2 and TMSNs-PO3 additional weight losses were observed, which can be considered as thermal decomposition of adsorbed Tacrolimus. Tacrolimus loaded MSNs showed the degradation of drug in same temperature range as pure Tacrolimus. This indicates that the drug is stable and in the same form after loading on the MSNs. The Tacrolimus loading was found 18.85 % in MSNs-C, 15.14 % in MSNsNH2 and 15.26 % in MSNs-PO3.

Journal of Chemical Health Risks

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JCHR (2024) 14(4), 406-419 | ISSN:2251-6727

Moreover, no endothermic melting peak of Tacrolimus was observed for drug loaded MSNs whereas, a clear endothermic peak (in range of 121 to 127 ◦C) is visible for free Tacrolimus. [22] This indicates that the drug is maintained in the amorphous form, and it is inside the pore. The Nitrogen (N2) adsorption–desorption analysis was performed to ensure the porosity and drug loading into the pores of MSNs. 3D structure and interconnected open network of MCM-48 type of nanoparticles make them very interesting carrier for drug delivery. Such structures have ability to enhance diffusion of drugs into and out of the pores, thus enhancing drug loading and controlled release of drug. The nitrogen physisorption results showed type IV isotherms for all the developed MSNs, which is characteristic of uniform mesoporous channels with narrow, cubic pores. The secondary mesopores show presence of nitrogen by process of capillary condensation which is identified by the narrow hysteresis at relative pressures near p/p0 < 1. H1 type of hysteresis loop at high relative pressure p/p0 < 1 indicates the nitrogen condensation into coarse mesopores while, the slope at intermediate p/p0 < 0.4 shows the nitrogen capillary condensation inside the mesopores. [23] The drug loaded MSNs demonstrated a significant decrease in the volume of nitrogen adsorbed reflected by lower surface area and pore volume. The measured BET specific surface area and adsorption average pore diameter for MSNs are mentioned in Table 2. The values for BET surface area and pore volume decreased after drug loading which further confirms the drug loading into the pores. FTIR spectroscopy was carried out to understand the interaction of Tacrolimus with MSNs surface at molecular level. Fig. 5a shows the presence of surfactant in the uncalcined silica (peak at 2930 cm-1) however, those peaks are absent when the MSNs are calcined or their surfaces have been modified with subsequent removal of surfactant by solvent extraction method. The major vibrational modes of silica surface groups were also observed. A comprehensive analysis of the spectra of drug loaded MSNs establishes existence and structural integrity of Tacrolimus molecules inside the silica pores. All in all, FTIR confirms the that Tacrolimus is in its original form after loading into MSNs. [24]

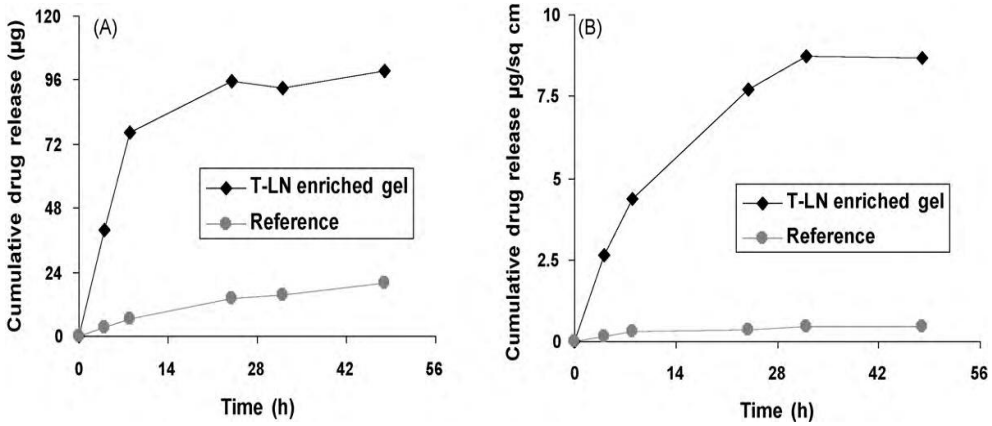


**Fig. 5.** In vitro drug release (A) and skin permeation (B) profiles.

### 3.2. Solubility studies

The solubility of Tacrolimus was estimated by preparing saturated solutions of Tacrolimus and TMSNs-C, TMSNs-PO3 and TMSNs-NH2 in water to reach the equilibrium concentration. Tacrolimus solubility was significantly enhanced in TMSNs-PO3 followed by TMSNs-NH2 (2.48 folds) and TMSNs-C (2.09 folds) compared to pure Tacrolimus. Hence, TMSNs-PO3 were selected for further studies and incorporation into gel formulation. The enhanced solubility of Tacrolimus is attributed to the confinement of Tacrolimus in mesochannels converting it into amorphous form. As described by the Ostwald-Freundlich equation, there is an increase in surface area with decreasing particle size which leads to increase in saturation solubility. Brij O20 was added to PBS to maintain sink condition during diffusion studies. Hence, it is important to check the solubility in the selected medium. It was observed that solubility of tacrolimus was significantly increased (~1000 fold) in presence of Brij O20 which further attest our hypothesis. [25]

### 3.3. Cytotoxicity studies and determination of expression of anti-inflammatory cytokines IL-6 by ELISA

Journal of Chemical Health Risks

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JCHR (2024) 14(4), 406-419 | ISSN:2251-6727

The *in-vitro* cytotoxicity of TMSNs-PO3 at various concentrations (500, 250, 100, 75, 50, 25, 10, 5 and 1 µg/mL) on HaCaT cells. There was no significant difference in cell viability suggest that the TMSNs-PO3 samples are biocompatible at all tested concentrations. It is well-known that Th2 immune response is pre-dominant in the lesions of AD and therefore an increased expression of Th2 cytokines such as IL-4, IL-5, IL-6, and IL-13, especially IL-6 has been found in the serum of AD patients. IL-6 is a multifunctional pro-inflammatory cytokine reported to be involved in inflammatory CD4+ T-cell proliferation and expansion exacerbating the inflammatory diseases. Therefore, blocking IL-6 action with an antibody targeting IL-6 receptor showed sustained efficacy in the patients with AD. As the immunosuppressant effects of Tacrolimus have long been used to target T-cells and treat AD, we sought to understand whether TMSNs-PO3 had also the similar potential in human keratinocytes. We therefore treated HaCaT cells with TMSNs-PO3 in absence or presence of LPS and measured the level of IL-6 in the cell supernatant. Our data showed that TMSNs-PO3 significantly ($p < 0.01$) reduced the expression of IL-6 induced by LPS. Although we did not observe any significant differences between Tacrolimus and TMSNs-PO3, treatment with TMSNs-PO3 tended to have better efficacy in the LPS-induced keratinocyte inflammation (Fig. 7b). As expected, Tacrolimus, MSNsPO3, or TMSNs-PO3 alone do not have any effect on IL-6 expression. Thus, topical delivery of Tacrolimus through negatively functionalised MSNs would be a better approach to treat AD. [26]

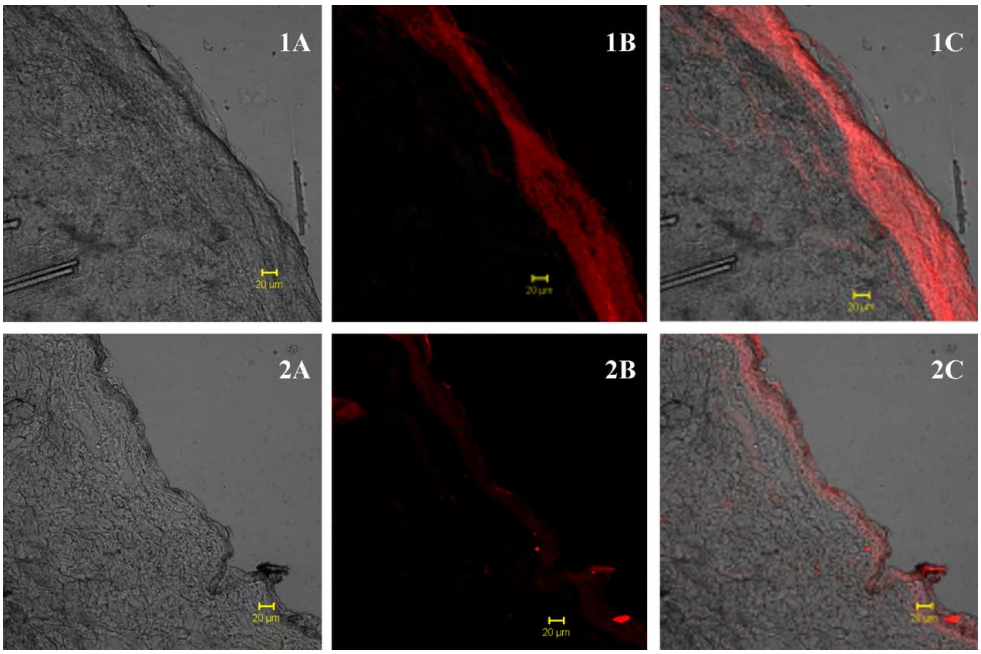


**Fig. 6.** Confocal laser scanning microscopy images showing cutaneous uptake and distribution of nanoparticles in the skin; bright field (A), detection of nile red labeled nanoparticles (B) and overlap of these two images (C); 1 denotes skin treated with T-LN and 2: reference.

### 3.4. Characterization of MSN enriched gel

Carbopol gel (1.25 % w/v) base was selected to incorporate the TMSNs-PO3 and free Tacrolimus. Prepared TMSNs-PO3 were embedded in to carbopol gel base formulation as it possesses shear thinning properties required for topical formulation to provide ease of application when applied with maximum coverage of the area. The consistency o the substance plays an important role in controlling the drug permeation through skin whereas viscosity influence spreadability, skin feel and penetration of incorporated actives across the skin. The measurement of viscosity of TMSNs-PO3 gel and Tacrolimus gel did not show notable different values when measured in a rage of temperature of (8–40 ◦C) indicating stability of viscosity of formulations over a range of temperature. Hence, the prepared gel will form thin film on the skin. The obtained spreadability value for TMSNs-PO3 was 11.2 ± 0.2 g.cm/s and 10.38 ± 0.41 g.cm/s for Tacrolimus gel which indicate that the formulation can be applied easily without any friction.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(4), 406-419 | ISSN:2251-6727

Therefore, the use of MSNs does not affect the gel characteristics evident from similar viscosity between gel with and without MSNs. pH plays the crucial role in designing topical formulations which can prevent skin irritation. Inappropriate pH can cause severe irritation, burning, and erythema at the site of application. In this study, the pH value of TMSN-PO3 gel was found to be 6.92 ± 0.50, thus confirming the biocompatibility for its dermal application and will not cause any stimulation to the skin. [27]

### 3.5. In-vitro drug release study

The *in-vitro* release profile of TMSNs-PO3. The increase in solubility of Tacrolimus from TMSNs-PO3 is evident from the *in vitro* drug release results in which PBS was used as the dissolution medium. After 30 min, TMSNs-PO3 gel exhibited 66.28% drug dissolution whereas pure Tacrolimus released only 48.50% over the same period. The rate of drug release for Tacrolimus alone was 55.01 % after 24 h, which was much slower than TMSNs-PO3, which had a better release profile of 73.29 % after 24 h. The increased dissolution rate of Tacrolimus from TMSNs-PO3 could be attributed to the amorphous state of Tacrolimus within cubic pores of MCM-48 and the higher surface area provided by colloidal nanoparticles resulting faster diffusion and release.

### 3.6. Ex-vivo skin permeation and skin retention study

Topically administered products are aimed to achieve desired skin penetration and drug localization to accomplish a site-specific therapeutic response. *Ex-vivo* study was performed to evaluate the applicability of MSNs as topical drug delivery carrier for Tacrolimus. The amount of Tacrolimus quantified in the skin 24 h after the application. For the experiments either TMSNs-PO3 gel (0.1 % w/w) or Tacrolimus gel (0.1 % w/w) were employed. The comparative analysis of permeation was done, and the results showed that the lower permeation of Tacrolimus was observed in case of TMSNs-PO3 gel (13%) and Tacrolimus gel (11%) which may be due to the selective permeability and the hindrance by the SC. These results represent that the permeation of Tacrolimus was minimal with both the gel formulation. The amount of Tacrolimus being delivered to various layers of skin was estimated by comparing the results of drug retention for TMSNs-PO3 gel and Tacrolimus gel. Results showed that the amount of Tacrolimus retained in the skin was (74.64 ± 0.9 %) in case of TMSNsPO3 gel whereas it was only 36% in case of Tacrolimus gel confirming our hypothesis. This could be due to increased surface area provided by nanosized particles and improved solubility. These findings suggest that TMSNs-PO3 has shown higher drug deposition in the skin and has the potential for the target-specific delivery of Tacrolimus in the management of AD. [28]

### 3.7. In-vivo studies

Skin irritation caused by topical products limits their use and acceptability among patients. Topical formulations are designed to deliver drug at the targeted layer of the skin, to achieve significant drug retention, and skin penetration to a certain degree, all while remaining non-irritating. Principle of Draize primary skin irritation test was used to perform skin irritation study on the rat's skin. The incorporation of Tacrolimus and TMSNs-PO3 in to carbopol gel base reduces the likelihood of direct contact of the Tacrolimus with the skin layers which further prevents the irritation. As expected our results show that the erythema score of the TMSNs-PO3 gel, Tacrolimus gel and control gel was zero compared to 1% formalin solution, which confirms the safety of our formulations. The irritation scores of the TMSN-PO3 gel were found to be lower compared to the Tacrolimus gel which could be due to the incorporation of Tacrolimus inside the pores of MSNs. Thus, it was concluded that the TMSNs-PO3 gel was non-irritant can provide benefits regarding improved patient compliance and acceptability for topical delivery of Tacrolimus. [29] It is expected that the potential and *in-vivo* efficacy of a formulation is governed by the rate at which the drug penetrates the skin and is retained at the site of inflammation. To confirm this, therapeutic efficacy of TMSNs-PO3 gel was evaluated *in-vivo* using hapten-induced murine model of AD in BALB/c mice. In the present study, we compared the effects of TMSNs-PO3 gel and Tacrolimus gel on ameliorating AD-like clinical symptoms in mice (n = 6). The clinical features of mice ear after the treatment with different formulations. After the sensitization and challenge with DNFB solution, the animals showed increase in severity of dryness and AD clinical symptoms including erythema, scarring and erosion. These symptoms were alleviated at different level after the treatment with TMSNs-PO3 gel and Tacrolimus gel in comparison with diseased control group. The ear swelling revealed the inflammation mediated by DNFB. The ear thickness was measured to evaluate ear swelling. [30] The ear swelling and thickness were significantly reduced with TMSNs-PO3

Journal of Chemical Health Risks

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JCHR (2024) 14(4), 406-419 | ISSN:2251-6727

gel treatment compared to the Tacrolimus gel treatment and disease control group. Histological examination was also performed to assess the therapeutic effectiveness of TMSNs-PO3 gel. Portrays that distinct epidermal hyperplasia, acanthosis, high number of infiltrated inflammatory cells and hyperkeratosis were observed with disease control group. Multiple dose application of DNFB led to these pathological features in response to the highest grades of allergic inflammatory reaction beneath the skin. It was observed from the images that keratinized epidermal layer and inner epidermal layers have been separated from each other and that may have caused severe itching. The group treated with 0.1 % w/w Tacrolimus gel showed fewer number of infiltrated inflammatory cells. The group treated with TMSNs-PO3 gel showed minimal hyperkeratosis and low degree of infiltration of inflammatory cells and acanthosis confirming our hypothesis. [31]

## 4. Conclusion

The MSNs in the desired size range of 200 nm- 300 nm were successfully synthesized and their surface was functionalized using positively and negatively charged groups. Surface functionalization was confirmed using characterization techniques like zeta potential, TGA, porosity analysis and specific surface area measurements. Tacrolimus was successfully loaded in the prepared MSNs and the impact of surface functionalization of the MSNs on the solubility of Tacrolimus was studied. Tacrolimus solubility was enhanced by 7 folds when loaded into the phosphonate functionalized MSNs. The amount of Tacrolimus loaded in the MSNs was determined in terms of qualitative and quantitative means by TGA and FT-IR analysis respectively.

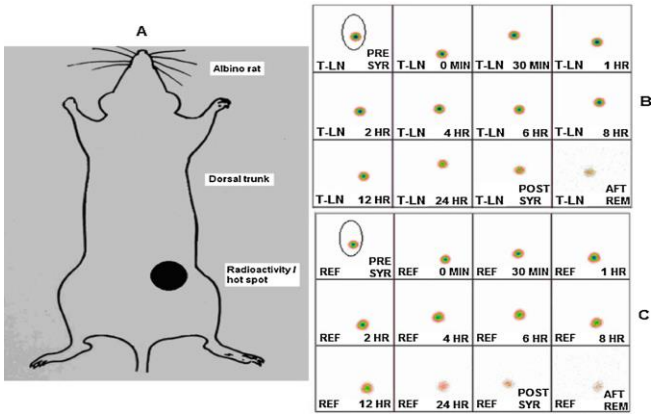


**Fig. 7.** Static whole body g-scintigraphic images procured after periodic time intervals for 24 h after topical application (each scintigram is taken over a frame time of 60 s); (A) placement of radiolabeled formulation on the depilated dorsal trunk of albino rat; (B) T-LN enriched gel and (C) reference.

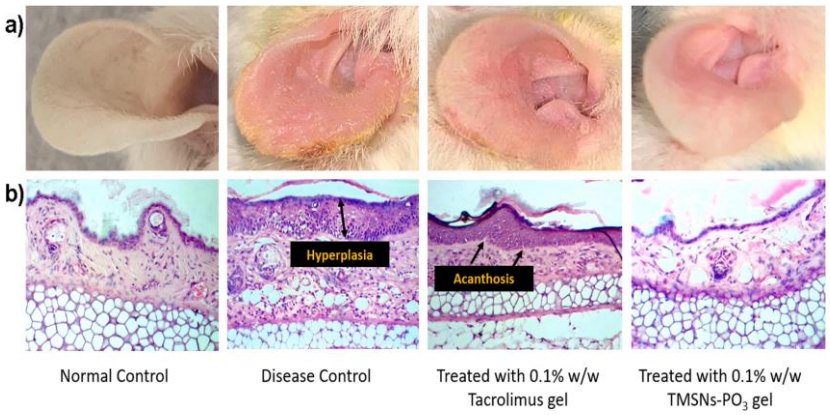


**Fig. 8.** *In-vivo* efficacy studies on animal model of atopic dermatitis. a) Representative images of ears of BALB/c mice b) histological evaluation by hematoxylineosin staining (H&E) of ear tissue.

Diffusion studies showed the improved release from the TMSNs-PO3 gel compared to the Tacrolimus gel. *Ex vivo* skin permeation results also validate the selection of MSNs as a carrier for topical delivery of the Tacrolimus by retaining significantly more Tacrolimus within skin. *In vivo* studies indicated that Tacrolimus loaded MSNs gel have the potential to improve the topical bioavailability of the drug. Hence, it could be concluded that MSNs seems a very promising approach for topical drug delivery of hydrophobic drugs such as Tacrolimus.

**Declaration of competing interest**

The author declares no conflict of interest, financial or otherwise.

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