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Stability and Safety Analysis of Statin-Loaded Nano-films for the Treatment of Diabetic Wound
(Analisis Kestabilan dan Keselamatan Filem Nano Sarat Statin untuk Rawatan Luka Diabetes)

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disease that delays the regular stages of the wound's healing process due to delayed inflammatory stages. Due to foot pressure points, chronic foot wounds are ultimately considered the primary cause of lower leg amputation. Diabetic patients have vascular dysfunction and neuropathy, leading to inadequate oxygen supply to the wound area. Statins have a crucial role in the regulation of angiogenesis that could increase vascular endothelial growth factor (VEGF) synthesis. By offering a localized treatment approach while minimizing systemic side effects associated with oral medication, this study aimed to develop statin-loaded nanofilms to determine their stability and safety among healthy individuals as a potential procedure for diabetic wound healing. Simvastatin (SIM) loaded nanofilms formulations (F1-F10) were prepared using the solvent casting method. The formulation was optimized based on tests such as physical appearance, tensile strength, microscopic photographs, morphology, and drug content uniformity. ICH guidelines were followed to determine various parameters (physical appearances, tensile strength, microscopic photographs, morphology, and drug content uniformity) for six-month stability study at three different storage conditions. Safety analysis of the nanofilms was performed on healthy human skin using the Draize skin irritation test. Results showed F7 formulation was considered an optimized formulation as well as stable through the storage period at 4 ± 2°C, 25 ± 2°C, and 40 ± 2 °C. Furthermore, Primary Irritation Index results (PII was 0 showed no irritation in case and control groups) indicate its safety and biocompatibility to skin. Thus, the optimized statin-loaded nanofilm is stable, safe, and non-toxic, which may be used as a potential diabetic wound healing agent.

Keywords: Nanoparticles; polymeric drug carrier; safety analysis; targeted drug delivery; wound healing

ABSTRAK

Diabetes mellitus (DM) adalah penyakit metabolik yang melambatkan peringkat biasa proses penyembuhan luka disebabkan oleh tahap keradangan yang tertangguh. Disebabkan oleh titik tekanan kaki, luka kaki kronik akhirnya dianggap sebagai punca utama amputasi kaki bawah. Pesakit diabetes mempunyai disfungsi vaskular dan neuropati yang membawa kepada bekalan oksigen yang tidak mencukupi ke kawasan luka. Statin mempunyai peranan penting dalam pengawalan angiogenesis yang boleh meningkatkan sintesis faktor pertumbuhan endotelium vaskular (VEGF). Dengan menawarkan pendekatan rawatan setempat sambil meminimumkan kesan sampingan sistemik yang berkaitan dengan ubat oral, kajian ini bertujuan untuk membangunkan nanofilem yang dimuatkan statin untuk menentukan kestabilan dan keselamatan mereka dalam kalangan individu yang sihat sebagai prosedur yang berpotensi untuk penyembuhan luka diabetes. Formulasi nanofilem yang dimuatkan simvastatin (SIM) (F1-F10) telah disediakan menggunakan kaedah tuangan pelarut. Formulasi dioptimumkan berdasarkan ujian seperti penampilan fizikal, kekuatan tegangan, gambar mikroskopik, morfologi dan keseragaman kandungan ubat. Garis panduan ICH diikuti untuk menentukan pelbagai parameter (penampilan fizikal, kekuatan tegangan, gambar mikroskopik, morfologi, dan keseragaman kandungan ubat) untuk kajian kestabilan enam bulan pada tiga keadaan penyimpanan yang berbeza. Analisis keselamatan nanofilem telah dilakukan pada kulit manusia yang sihat menggunakan ujian kerengsaan kulit Draize. Keputusan menunjukkan

formulasi F7 dianggap sebagai rumusan yang dioptimumkan serta stabil melalui tempoh penyimpanan pada 4 ± 2 °C, 25 ± 2 °C dan 40 ± 2 °C. Tambahan pula, keputusan Indeks Kerengsaan Utama (PII ialah 0 menunjukkan tiada kerengsaan dalam kes dan kumpulan kawalan) menunjukkan keselamatan dan biokeserasiannya kepada kulit. Oleh itu, nanofilem dimuatkan statin yang dioptimumkan adalah stabil, selamat dan tidak toksik yang boleh digunakan sebagai agen penyembuhan luka diabetes yang berpotensi.

Kata kunci: Analisis keselamatan; nanozarah; pembawa dadah polimer; penghantaran dadah yang disasarkan; penyembuhan luka

Introduction

DM is a condition that has grown in severity during the last 20 years and has become a global health concern. Around 30 million individuals had diabetes in 1985, which rose to 285 million by 2010. A recent report issued by the International Diabetic Federation in 2019 showed that the number of patients affected by diabetes has increased to 463 million. The estimated number of people living with diabetes in 2045 is about 700 million (Das et al. 2020).

DM posed a great impact not only on the length but also on the quality of life of patients. It also creates a considerable financial burden for patients. According to the International Diabetes Federation 2017, the diabetes atlas placed Pakistan among the top 10 of the total 221 countries that are estimated to account for 7.5 million total cases of diabetes (Chandrasekaran, et al. 2023). In the released country profiles by World Health Organization (WHO), it was claimed that Pakistan lacks a national approach to diabetes involving an implementation policy, nationwide diabetes regulations, and a national diabetes record.

Obesity is one of the major contributing factors behind Pakistan's diabetes epidemic, including both urban and rural people and is currently prevalent among young adults at 15%. According to various studies, chronic diabetes is associated with several complications, such as peripheral vascular disease affecting 5.3% and cerebrovascular illness affecting 4.5%. Diabetic Foot Ulcers DFU's are a prevalent DM complication, which has exhibited an increasing tendency in previous eras. Given the present prevalence of diabetes, it is projected that 1.09 million persons in Pakistan also have DFU (Golkar et al. 2023). As per the estimation, 15% of people suffering from DM experience DFU simultaneously (Monteiro-Soares et al. 2020). DFU contributes to 20% of hospitalization in patients suffering from diabetes. If proper care of the wound is not done, that leads to serious issues like gangrene and amputation. About 50-70% of all lower limb amputations is considered to be caused by DFU (Rastogi et al. 2020). DFU is also associated with considerable mental and physical anguish and performance, including financial deficits that reduce the quality of life (Yazdanpanah, Nasiri & Adarvishi 2015).

Statins have recently shown various pleiotropic benefits apart from their cholesterol reduction, thus currently regarded as a new potential therapeutic method for several diseases, like sepsis, anti-inflammatory, maintenance of immune functions, and wound healing (Daliri, Johnston & Sahebkar 2023). When statins interfere with the synthesis of cholesterol in the body, they use all the intermediatory substances-isoprenoids. As a result, the depletion of isoprenoids leads to processes like anti-inflammatory and immunomodulation. Statins also promote the wound-healing process in diabetic patients by boosting the formation of new blood vessels with lymph angiogenesis (Penton et al. 2023). Topical statin treatment has been proposed as a viable alternative to oral statin dosage to determine whether statins benefit diabetic wound healing.

SIM has been discovered to have a wound-healing property that helps produce different vascular endothelial growth factors, releasing them in the wound area. New growth factors at the wound site help in the production of new blood vessels to increase and maintain the blood flow at the wound area to help heal the wound. SIM has been proven to have antibacterial activity, reducing Gram-positive bacteria, making it a viable substitute for some of the well-known antibiotics (Gupta, Quadros & Momin 2023). Simvastatin has limited bioavailability due to its inability to dissolve in water, making it the perfect candidate for topical formulation. To increase absorption, better therapeutic effectiveness, and skin permeability, it was developed as a nano formulation (Aly et al. 2019; Sameh et al. 2018; Yazdanpanah, Nasiri & Adarvishi 2015).

Existing medicines can be reformulated into a new drug administration system with superior clinical and target-specific properties to the current ones (Gayathri et al. 2023). Furthermore, certain oral formulations were discovered to be beneficial *in-vitro* while ineffective when given an *in-vivo* dose, owing to enzymes prevalent in the gastrointestinal system, so new and modified systems were required to maintain the required efficacy (Gayathri et al. 2023; Raval et al. 2019; Sameh et al. 2018).

Nanoparticles are currently being given great attention in providing therapeutic drug delivery. There has been improvement in the angiogenesis process due to the administration of nanoparticle-based drug delivery systems (Tiwari et al. 2023). The developed nanoparticles can be further integrated into biomaterials and in scaffolds to form new nanocomposites that also possess antibacterial and pro-angiogenesis properties. They can be utilized as gene transport vectors to affect internal gene expression and protein manufacturing during wound healing. They can also affect wound healing by altering collagen accumulation and reconfiguration (Naderi et al. 2018; Raval et al. 2019; Tiwari et al. 2023). Numerous non-toxic, bio-compatible and naturally and synthetically prepared polymers are used for drug delivery (Madawi et al. 2023). Amongst all chitosan (CS) is naturally occurring, approved by the Food and Drug Administration (FDA) polymer that exhibits wound healing capability (Ebhodaghe 2022).

In the previous study, our laboratory successfully fabricated and optimized SIM-encapsulated chitosan nanoparticles (CS-NPs) loaded polymeric film (Tufail et al. 2022). Although SIM-CS-NPs loaded polymeric film has wound healing efficacy in diabetes-induced rat models, it has not been evaluated for its safety and tolerability in human skin. Hence, in this study, polymeric film was developed and evaluated for its safety in human skin. In addition, the stability of this nanoparticle-loaded polymeric film was evaluated based on its physical appearance, tensile strength, morphology, and drug content uniformity at 4 ± 2 °C, 25 ± 2 °C, 40 ± 2 °C.

Materials and Methods

SIM was obtained from Unexo Pharmaceuticals Lahore. Lower molecular weight (LMW) chitosan (CS), hydroxypropyl methylcellulose (HPMC), Sodium Alginate (SA) and sodium tripolyphosphate (TPP) were purchased from Sigma Aldrich. Sodium alginate, glycerin, acetic acid, and ethanol were of analytical grade.

Nanoparticle preparation using ionic gelation method

SIM-loaded CS-NPs were prepared by the following ratios (Table 1). One mg/mL CS solution was prepared using 1% acetic acid (pH 5.0), followed by stirring overnight at room temperature (25 °C). TPP solution (1 mg/mL) was formulated using pure distilled water. Both CS and TPP solutions were filtered by a syringe filter (0.45 µm Millipore, USA) to remove any undissolved particles. The SIM solution (100 mg) was prepared separately in 5 mL ethanol and then added to the CS solution, followed by stirring at 1200 rpm for 4 h. TPP solution was then added dropwise over SIM-CS solution at 1200 rpm at 25 °C (room temperature) for 12 h. Light turbidity of NP formulation was visualized. The pellet was collected via centrifugation at 9000 rpm for 30 min. The pellets were freeze-dried for further analysis and formulation development (Wang et al. 2019).

TABLE 1. Compositions of SIM-loaded chitosan nanoparticles (SIM-CS-NPs)

Sr. No	Formulation	Chitosan: TPP (mg)	SIM (mg)	Water (mL)
1	M1	1:1	100	100
2	M2	2:1	100	100
3	M3	1:2	100	100
4	M4	2:2	100	100

DRUG LOADING ESTIMATION

Drug-loaded nanoparticles were centrifuged at 12000 rpm for 30 min. After centrifugation, the supernatant was collected and analyzed for drug content using a UV-VIS spectrophotometer (Shimadzu 1800) at 283 nm (Leonetti et al. 2021). Encapsulation efficiency, drug loading, and percentage yield were calculated using the following formulas,

$$EE\ (\%) = \frac{weight\ of\ drug\ in\ nanoparticles - initial\ drug\ weight}{initial\ drug\ weight} \times 100$$

$$\text{DL}\ (\%) = \frac{\text{drug weight in nanoparticles}}{\text{nanoparticles weight}} \times 100$$

$$\text{Yield}\ \%\frac{\text{w}}{\text{w}} = \frac{\text{weight of dried nanoparticles recovered}}{\text{weight of drug} + \text{polymer weight}} \times 100$$

DRUG RELEASE STUDY

The dialysis membrane method was used to understand nanoparticle drug release (Azevedo et al. 2014). Nanoparticle solutions were placed in a dialysis membrane to determine drug release from nanoparticles. The experiment was conducted at 37 °C, and phosphate-buffered saline (PBS) media at pH 7.5 was used. The drug release process is as follows: 50 mg of SIM-loaded nanoparticles were redispersed in 5 mL phosphate buffer solution with a ratio of ethanol 9:1 at pH 7.5. Furthermore, the solution was placed in a dialysis membrane (molecular weight cut-off 35000 Da Cell-Sep H1, membrane filtration product, USA). The membrane was then sealed and placed in PBS solution; the entire assembly was held in a 37 °C magnetic hot plate and mixed continuously at 100 rpm. After every sample collection (approximately 3 mL), the solution quantity was refilled to maintain sink condition. SIM-CS-NPs' drug content was measured using a UV-VIS spectrophotometer (Shimadzu 1800) (Afshar et al. 2020).

NANOFILM PREPARATION

Nanofilms were prepared using the solvent casting method (Tufail et al. 2022). Polymeric solutions of two different polymers (HPMC and SA) were prepared separately by soaking, followed by dissolving in distilled water until a clear solution was achieved. Glycerin was added as a plasticizer to increase the flexibility of these prepared nanofilms (Chandra Hembram et al. 2016). HPMC 3 g was the standard for more combinations, and different ratios were developed using SA to develop the desired smooth, easy-to-use nanofilm. A combination of two polymers was made in different ratios, as in Table 2.

The prepared polymeric solutions were sonicated to remove trapped air-bubble followed by the addition of CS-NPs colloidal solution. The resulting solution was placed on the magnetic stirrer for 3-4 h. to get the desired suspension. It was then further placed in a sonicator to remove the air-bubble. Petri dishes of 90 mm were used to pour the solution. These petri dishes were lubricated with pure glycerin to help to peel off the patch. Petri dishes were incubated for 48 h at 38 °C to get the dry films. The films were peeled off from Petri, dished, wrapped in butter paper, and further sealed in an aluminium pouch to maintain their integrity (Wadher et al. 2023).

TABLE 2. Different combinations of HPMC and SA along with the glycerin

Formulations	HPMC:SA	Water (mL)	Glycerin (g)	Flexibility
F1	1:1	100	2	No
F2	1:2	100	2.5	Yes
F3	1:3	100	3	No
F4	1:4	100	3.5	No
F5	1:5	100	4	No
F6	2:2	100	2	Yes
F7	2:1	100	2.5	Yes
F8	3:1	100	3	Yes
F9	4:1	100	3.5	Yes
F10	5:1	100	4	No

STABILITY STUDY

Stability studies were performed as per ICH guidelines using three different storage conditions (4 ± 2 °C, 25 ± 2 °C, 40 ± 2 °C). Statin nanofilms were placed in a well-closed container for six months. Analysis was performed after 1, 3, and 6 months for the physical appearance, tensile strength, microscopic photographs, morphology, and drug content uniformity.

Physical Appearance

Clarity and smoothness were checked in the prepared nanofilms. All the prepared films were placed at three different storage conditions were checked for their physical appearance (DCruz, et al. 2022).

Tensile Strength

The highest stress is imposed to a point where the patch breaks are referred to as tensile strength. The film's tensile strength was evaluated using tensile strength testing equipment. The apparatus's jaws were fitted with rectangular patch strips measuring 2 × 2 cm. At a rate of 4 mm/min, the weight on the patch was steadily elevated to a maximum limit until it broke (Yao et al. 2022). The tensile strength was determined using.

$$Tensile\ strength = \frac{Load\ at\ break}{Sectional\ area}$$

MICROSCOPIC PHOTOGRAPHS

Dino lite® digital microscope was used to examine the nanofilms under the microscope. These nanofilms were shaped into a spherical form with a surface area of 2 cm^2 and examined. The photographs were taken at a magnification of 200x.

MORPHOLOGY

The morphology of nanoparticles was observed using a scanning electron microscope (SEM). SIM-CS-NPs loaded polymeric patch of around 2 × 2 cm was cut and placed on the silicon chip to check the morphology of the loaded nanoparticles for months. Morphology was checked at 1st, 3rd, and 6th month at three different storage conditions (4 ± 2 °C, 25 ± 2 °C, 40 ± 2 °C) (Singh et al. 2020).

DRUG CONTENT UNIFORMITY

Patches measuring 2×2 cm in diameter were selected from various locations among the stored patches. Each nanofilm was dissolved in the buffer PBS pH 7.4 in a flask total of up to 100 mL. 1 mL was withdrawn and diluted to 10 mL with buffer solution. A wavelength of 238 nm was set to determine the absorbance of the dissolved patch. The percentage drug concentration was determined using the calibration curve, and the procedure was performed three times with three patches of each stored formulation.

CLINICAL SAFETY ANALYSIS

Study objective

This study's primary objectives were to assess the safety of SIM-NP nanofilm on healthy human skin and to investigate the local harmful effects of the nanofilm formulation using the Draize skin irritation test (Afshar et al. 2020; Bolton 2016; Dcruz et al. 2022; Singh et al. 2020; Yao et al. 2022). The developed nanofilms were cut to a proper size (2×2 cm) and placed on the adhesive (Nichiban tape) to apply on human skin.

Study Population

The sample size for this study was twenty healthy human adults. Twenty healthy adult volunteers from 18-40 years old were enlisted in this study based on the inclusion and exclusion criteria listed in the table below (Table 3). Human research was conducted following the Institutional Review Committee for Biomedical Research, University of Veterinary and Animal Sciences, Lahore, Pakistan (177/IRC/BMR). All participants received written informed consent (Bibi et al. 2022). Volunteers were divided into two groups, the control positive group and the control negative group, based on the inclusion and exclusion criteria in Table 3. The control positive group was applied drug-loaded nanoparticle film, while the control negative group was the film without drug-loaded nanoparticles.

TABLE 3. Inclusion and exclusion criteria for clinical study

Inclusion criteria	Exclusion criteria
1. Men and women 18 years old or above	1. Females with pregnancy and lactation
2. Willing to participate and signed consent	2. Patient under 18years
3. On no regular medical treatment	3. Mentally or neurologically disable

Study Design
The research was carried out following good clinical practice guidelines. Skin observation was part of the study examination using the Draize skin irritation method, including the erythema and oedema scoring, as mentioned in Table 4. All the enrolled subjects will be checked for irritation scoring per the primary irritation index scoring, as mentioned in Table 5.

Study Period
Both groups were asked to apply the film on their skin for 6 h (Mahmood & Akhtar 2013). The skin reactions were assessed after 1st, 2nd, 6th, and 24th h. Erythema and oedema were assessed using a visual grading system that ranges from 0 to 4, with 0 indicating no erythema and 4 indicating significant erythema.

Data Analysis
After running experiments with optimization intention, statistical analysis was performed, and the results were displayed as ±3 SD. To establish the deviation and test hypothesis, the Statistical Package for the Social Sciences (SPSS) software was used, and the data were analyzed using the analysis of variance (ANOVA) test, including student's t-test and Tukey's post-hoc test where appropriate at a level of significance of $p<0.05$ (Ramzan et al. 2021; Sinha et al. 2021).

TABLE 4. Draize skin irritation scoring

Erythema/Escher formation	Value	Edema formation	Value
No erythema	0	No edema	0
Very slight erythema (barely visible)	1	Very slight edema (barely visible)	1
Well-defined erythema	2	Slight edema (edges well defined by definite raising)	2
Moderate to severe erythema	3	Moderate edema (raised to 1 mm)	3
Severe erythema	4	Severe edema (raised more than 1 mm)	4

TABLE 5. Primary irritation index scoring

Primary irritation index (PII)	Irritation score
Non-irritant	0-0.4
Mild irritant	0.5-1.9
Moderate irritant	2.0-4,.9
Severe irritant	5.0-8.0

Results and Discussion

Physicochemical Characterization of Nanoparticles

SIM-loaded CS-NPs were fabricated by the standard ionic gelation method with some modifications (Calvo et al. 1997). SIM loaded CS-NPs were optimized by using different concentrations of CS and TPP and keeping the concentration of SIM constant at 1200 rpm. The particle size of the resulting NPs affected by the complexation of high negatively charged polyanionic (six ionic group) TPP and positively charged polyanionic (quaternary amine group) of CS, at pH 5.0 and room temperature (Algharib et al. 2022). Drug loading capacity, entrapment efficiency, and drug release of the SIM-loaded NPs were calculated using mean and standard deviation (mean ± S.D., n = 3) (Table 6). In general, increases in particle size and zeta potential are affected by changes in the ionic interaction of CS and TPP (Hoang et al. 2022). Among four different formulations M1 ratio was selected for further tests due to its particle size (192 ± 11 nm). The NPs demonstrated an average size of around < 250 nm, making them desirable to be easily absorbed through the skin (Ta et al. 2021). The stability of a colloidal solution depends on its zeta potential. Zeta potential (+38 ± 4.28 mV) was considered to be the most stable dispersion to prevent particle aggregation (Cai et al. 2022). PdI of nanoparticles was found in the range of 0.2 ± 0.01 - 0.5 ± 0.04. Whereas, the M1 formulation showed a PdI value of 0.2 ± 0.01, indicating the monodistribution of nanoparticles in this formulation, making it more desirable. M1 formulation was the best one having drug loading and entrapment efficiency 49.9% and 99.8%. The CS and TPP complexation influence the size of particles and zeta potential. Whenever the ratio of both CS and TPP changes, the ionic interaction also changes, resulting in different particle sizes. A similar pattern in nanoparticle formulation involving electrostatic interactions was observed when the results were compared with existing literatures. However, specific polymer percentages, pH levels, and other formulation parameters used in various studies may influence the exact values and outcomes (Begines et al. 2020). This is unique to our findings as we achieved a desired particle size range (<250 nm), which is important for better skin absorption and an effective drug delivery system. Besides this, the high entrapment efficiency of developed nanoparticles indicated that almost all drugs were loaded in developed nanoparticles. Moreover, high loading efficiency is important since it reduces drug loss during manufacturing, making it cost-effective while maintaining therapeutic efficacy.

Nanofilm Preparation

By adopting the most basic technologies, solvent casting method, film preparations containing nanoparticles provide a scalable and cost-effective way of generating nanoparticles loaded film as solid dosage forms. The SIM-containing nanoparticles were then integrated into a polymeric film made up of a combination of HPMC, and SA. The change in the concentration of polymers did not affect on physical parameters of the film. The thin and homogenous thickness of the film is required for cutaneous application (Tiwari & Pathak 2023). After the film was dried, it is essential to observe the presence of any air-bubble or sedimentation as well as the agglomeration of solid particles to maintain the homogeneity of film (Karki et al. 2016). Films peeled off after drying. Peel ability of the formulations F6 and F7 formulations were better as compared to others. In comparison to other films with poor hydrogen bonding, nanofilms with a high hydrogen connection involving polymers with nanoparticles will have better physical characteristics (Saleh et al. 2020). Developed films with a stronger hydrogen bonding were preferred as they were reproducible quickly and exhibited greater mechanical strength due to the intricate bonding with better stability and durability. The elasticity of the film in the presence of plasticizer (glycerin) was improved. F6 and F7 showed smooth texture, folding endurance, and acceptable elasticity. This will allow the handling of nanofilms through slicing and packaging, making it much easier. Rigid-surfaced films can potentially be irritating to the skin or wounds of patients (Cai et al. 2022; Sharma et al. 2020).

Stability Study

Physical Appearance

Nanofilms placed as per ICH guidelines using three different storage conditions (4 ± 2 °C, 25 ± 2 °C, 40 ± 2 °C) were checked for their physical appearance characteristics at 1, 3, and 6 months. Nanoparticle loaded film showed that it is a stable formulation. For the time of 6 months, both 4 °C and 25 °C storage showed that films were clear, smooth, uniform, and flexible conditions making them acceptable for skin application. Whereas harsh conditions like 40 °C removed the moisture content making it more rigid, irregular, and broken one

(Schoenmaker et al. 2021). In the addition of plasticizers including glycerin, the mixture of polymers improves the flexibility and resilience of nanofilms. Nanofilms that are clear and flexible are preferred as the patients show better compliance towards them (Wang et al. 2021). All these stored patches were checked for clarity, smoothness flexibility, and uniformity and all the results are mentioned in Table 7.

Stored SIM-loaded nanofilms were found to retain the physical properties for 6 months. Stored formulations at 4 ± 2 °C and 25 ± 2 °C were found to be clear smooth flexible and uniform for 3 months. Testing done after the 6th month showed a decrease in the flexibility of the patch, but all the other characteristics were retained normally. Nanofilms stored at 25 ± 2 °C remained clear, smooth, flexible, and uniform for 6 months that were suitable and acceptable for skin application. Clarity, smoothness, flexibility, and uniformity retention after storage of 6 months at controlled temperatures (4 ± 2 °C and 25 ± 2 °C) showed the promising stability of these formulations and demonstrated their suitability for practical use (González-González et al. 2022). There was slightly less flexibility of formulation after 6 months, while all other properties remained intact, indicating a potential limit in long-term storage in terms of flexibility. Not only were the formulations found to be stable and durable throughout storage, but the results also showed that temperature maintenance during this time needs to be carefully monitored or assessed.

TABLE 6. Particle size, zeta potential, drug loading and drug entrapment efficiency of SIM-loaded CS-NPs

CS: TPP w/v	Particle size (nm)	Zeta potential (mV)	PDI	EE (%)	DL (%)	Drug release over 72 h (%)
M1	192 ± 11	+38 ± 4.28	0.234	99.82 ± 5	49.99 ± 4	90.84 ± 9
M2	225 ± 9	+49 ± 7.25	0.329	94.64 ± 4	41.01 ± 3	85.05 ± 6
M3	187 ± 13	+40 ± 6.45	0.546	90.16 ± 6	32.11 ± 5	78.81 ± 5
M4	258 ± 10	+58 ± 7.23	0.365	98.07 ± 3	27.00 ± 2	68.82 ± 7

TABLE 7. Physical appearance of stored nanofilm at various temperature following ICH guidelines

Storage conditions	4 ± 2 °C	25 ± 2 °C	40 ± 2 °C
At 0 month	Clear Smooth Uniform Flexible	Clear Smooth Uniform Flexible	Clear Smooth Uniform Flexible
After 1 month	Clear Smooth Uniform Flexible	Clear Smooth Uniform Flexible	Clear Rough Uniform Hard
After 3moths	Clear Smooth Uniform Flexible	Clear Smooth Uniform Flexible	Irregular Rough Rigid Hard
After 6 months	Clear Smooth Uniform Less flexible	Clear Smooth Uniform Flexible	Irregular Rough Rigid Broken

Tensile Strength

Nanofilms stored at three different storage conditions (4 ± 2 °C, 25 ± 2 °C, 40 ± 2 °C) were checked for their tensile characteristics after 1, 3, and 6 months. The parameter of tensile strength must be validated to understand the crack or breakage of the film. The value of the tensile strength ranges from 2.379 ± 0.39 to 2.325 ± 0.42 as mentioned in Table 8. Multiple time testing during the storage period showed that there was a gradual decrease in the tensile strength of the patches throughout 6 months during 4 ± 2 °C, and 25 ± 2 °C storage conditions ($p \leq 0.05$) (Sinha et al. 2021). The intermolecular interaction between the polymers and nanoparticles has led to the development of bonds. This forms a stronger network, ultimately materializing a more comprehensible film structure (Xi et al. 2013). Whereas, tensile strength was much of its lower value at 40 ± 2 °C. Likewise, the tensile strength of all the stored patches was evaluated using an auto-tensile tester to see whether they were mechanically strong enough to withstand the breakdown of the skin barrier and penetration. The tensile strength of the stored patches varied from 2.379 ± 0.39 to 2.325 ± 0.42 mpa. After the application of one-way ANOVA, there were significant differences between the formulations ($p \leq 0.5$) at both 4 °C and 25 °C, except 40 °C. Regarding high temperatures, it might be noted that tensile strength steadily declines over six months. This suggests that temperature significantly influences these patches' mechanical characteristics. As per the literature, this implies that the temperature-sensitive degrading process impacts the overall integrity of stored patches. The patch's continuous loss of strength highlights the need for better temperature management during storage to ensure sustainability, indicating their potential practical applications (Li et al. 2023).

Microscopy of Nanofilms

The microscopic morphology of the surface of nanofilms was observed using a dino-lite® microscope at different storage conditions after 1 month, 3 months, and 6 months as shown in Figure 1. Below a dark background, the films typically show homogeneous monoclinic crystal patterns, that even showed cluster formation of many nanoparticles together at different storage conditions throughout 6 months. The integrity of structures remains intact during storage, which is evident from the homogenous monoclinic structure observed throughout the storage duration. The literature showed that clustered nanoparticles tend to form agglomerated if storage conditions are inadequate. The structural stability must thus be ensured through proper control of storage conditions. Such findings emphasize strong crystalline patterns in films and demonstrate their structural stability, which is necessary for possible applications (Favela-Camacho et al. 2019).

SURFACE MORPHOLOGY SEM

SEM was used to examine the morphology of nanoparticle loaded film at three different temperature storage conditions at 1, 3 and 6-months Figure 2. SEM is known as an analytical technique that uses an electron beam to micro-analyze a sample. As a result, an enlarged picture of the specimen is displayed on the screen, allowing the morphological characteristics of the sample to be determined. Moreover, the size and shape-related features of this patch were identified using SEM analysis. The retention of particle integrity points to the potential benefits for long-term drug administration by these NPs, making them extremely encouraging for simvastatin's application as a sustained delivery system. After the SEM analysis at different magnifications on the stored

TABLE 8. Tensile strength(n/mm²) of nanofilms at three variable temperatures

Storage conditions	4 ± 2 °C	25 ± 2 °C	40 ± 2 °C
At 0 month	2.365 ± 0.32	2.375 ± 0.37	2.379 ± 0.39
1 month	2.345 ± 0.32	2.375 ± 0.37	2.125 ± 0.39
3 months	2.335 ± 0.22	2.345 ± 0.32	1.243 ± 0.32
6 months	2.315 ± 0.42	2.337 ± 0.52	0.695 ± 0.12

nanofilms, it showed that SIM-loaded nanoparticles showed the smooth and desired size of nanoparticles at both 4 °C and 25 °C temperature and no particle agglomeration was seen. Whereas it has been seen that particle lose their shape at 40 °C (Kittaneh et al. 2023). SEM pictures of different magnitudes showed that even after months, simvastatin-loaded nanoparticles were intact and remained stable in their shape. The outcomes highlight the significance of more research into the specifications of the encapsulation process and their impact on long-term stability, opening up possibilities for developing dependable and long-lasting drug delivery systems with improved efficacy and shelf-life (Guo et al. 2021).

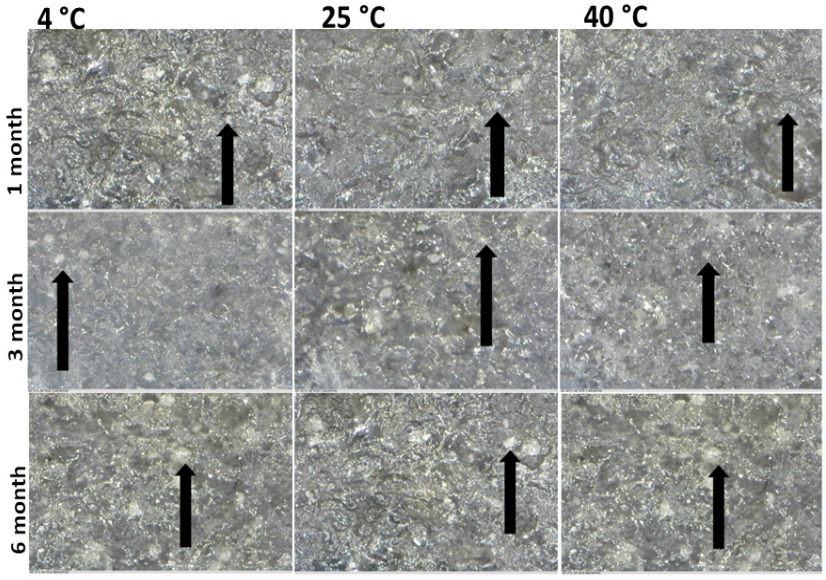

FIGURE 1. Microscopic morphology of the surface of nanofilms for 6 months different storage condition

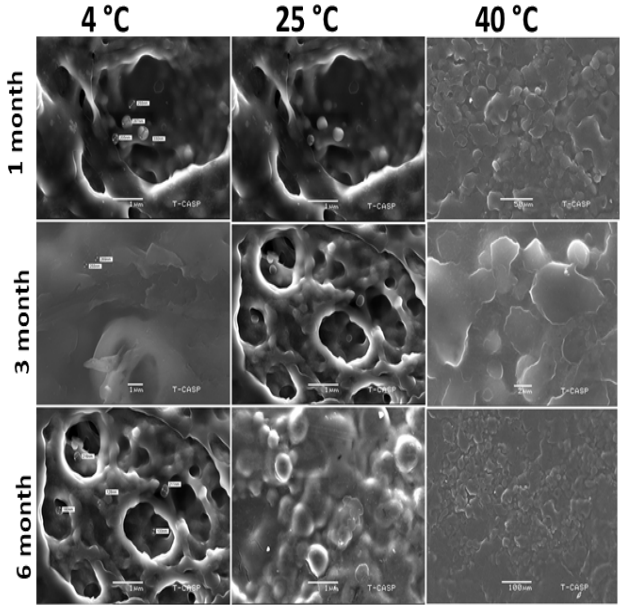

FIGURE 2. SEM analysis for 6 months at different storage temperatures

Drug Content Uniformity

The drug content of the simvastatin-loaded nanofilms was checked for the aging period of six months and the value was determined between 88% and 80% as shown in Table 9. Statistical analysis (One-way ANOVA) showed that the drug content of fresh and stored patches did not alter significantly ($P > 0.05$). As a result, the standard deviation values were relatively low, indicating that medication contents were uniform. Drug content uniformity was found between 88% and 80% during the storage time at three different storage conditions. It was once performed to verify the specifications for therapeutic products that rate the quality of stored nanofilms. We were able to guarantee that the strength of a medicinal product stays within predetermined acceptable limits due to analysis for content uniformity. According to the literature, the formulation's susceptibility to environmental conditions, such as temperature, humidity, and chemical interaction, was demonstrated by a decline in drug content uniformity after the storage term (González-González et al. 2022). Even though this formulation adhered to pharmaceutical standards, thorough consideration must be given to the practical implications to guarantee the medication's efficacy.

SAFETY ANALYSIS

Twenty healthy volunteers were separated into 2 groups: the study group and the control group, for an *in-vivo* irritation study. At different time intervals (1, 2, 6, and 24 h), irritation symptoms were assessed and a Dino-lite® microscope was used to check the area before and after the application of the SIM-NP patch as shown in Figure 3. The Draize skin irritation method was used to calculate the findings. Skin irritations are intricate biological processes that can range from rapid contact-induced acute reactions to persistent dermatitis. Chemicals can enter the skin by passing the intercellular lipid layer, transcellular with direct penetration via cornified cells, diffusely along hair follicles and sweat glands, or through the skin that has been damaged (Ruan et al. 2022). Corrosives are substances that can severely harm tissue and necrosis, leading to irreparable tissue damage. Acute irritants create inflammation after even a single use, whereas cumulative stressors bring irritation with repeated exposure. At the same time, irritants are compounds that have reversible effects on the skin (Sahu et al. 2021). It is advised to use a progressive testing approach for newly released drugs to gather reliable scientific information about their irritability (Jain et al. 2022). Standard Draize skin irritation scoring system was used for erythema and oedema evaluation, as well as the primary irritation index (PII) (Yong et al. 2019). The PII was determined under the supervision of a dermatologist by dividing the average erythema/edema score by the total of observations (Table 10).

Following 1, 2, 6, and 24 h of observation, all three individuals in the study group were assigned a 0 score, indicating that there was no redness or edema after application. Furthermore, the application site remained normal throughout the study period and no irregularities were discovered. The study's findings showed that neither the study nor the control group showed any evidence of irritation. Nanofilms proved non-irritating and safe for human use, whereas the control group, had made the same observation. For this study, the PII was zero, falling inside the non-irritant score range (0-0.4). As a result, the designed patch was determined to be non-irritating, as well as safe, and have good biocompatibility for human skin application. Based on the findings and statistics, it was determined that the F7 formulation comprising SIM-NP with a combination of polymers provided the more promising outcomes and should be considered an optimized formulation that met all the requirements for the patch of biocompatibility that would be used for the effective treatment of diabetic wounds. Topical treatments against skin irritation have been subjected to similar grading techniques as the ones other studies, this research does not cause any discomfort, which means that these results are essential in assessing used in the present study that emphasize skin tolerance

Table 9. Percentage of drug content uniformity of stored nanofilms

Storage conditions	4 ± 2 °C	25 ± 2 °C	40 ± 2 °C
After 1 month	85%	88%	86%
After 3moths	84%	85%	82%
After 6 months	82%	84%	80%

assessments leading to irritation reactions. In line with the safety and acceptability of dermal products. These findings agree with prior investigations that advocate for careful examination of skin irritancy as an initial important stage for ensuring the reliability and suitability of topical preparations meant for human use (Hardwick et al. 2020).

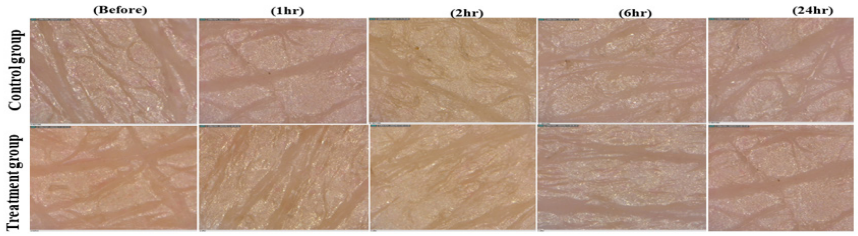

FIGURE 3. Before and after skin surface microscopic pictures of the control group and treatment group

TABLE 10. Skin irritation scoring observation

Human group name 1 h		Erythema/Edema score			
		2 h	6 h	24 h	
Study group	S1	0	0	0	0
	S2	0	0	0	0
	S3	0	0	0	0
	S4	0	0	0	0
	S6	0	0	0	0
	S7	0	0	0	0
	S8	0	0	0	0
	S9	0	0	0	0
	S10	0	0	0	0
Control group	C1	0	0	0	0
	C2	0	0	0	0
	C3	0	0	0	0
	C4	0	0	0	0
	C5	0	0	0	0
	C6	0	0	0	0
	C7	0	0	0	0
	C8	0	0	0	0
	C9	0	0	0	0
	C10	0	0	0	0
Total score		0	0	0	0
Mean score		0	0	0	0
PII		0			

Conclusions

Thus, the current study demonstrated the effective development of a nanofilm-based delivery method including simvastatin-loaded nanoparticles for local diabetic wound repair. Throughout the six months, the formulation demonstrated excellent skin application and storage qualities. The findings showed that SIM-loaded nanofilms might be safely stored without losing their properties. The findings of the safety studies demonstrated that nanofilm is safe to apply to human skin, but more research is needed to fully understand their actual potential as wound-healing formulations. As a result, nanofilms loaded with a statin could be safely useful as a topical formulation for diabetic wound treatment.

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