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# Formulation Development and Characterization of Nanoemulsion-Based Formulation for Topical Delivery of Heparinoid

Pooja Bakshi [1], Ying Jiang [1], Takahiro Nakata [2], Junji Akaki [2], Nobuya Matsuoka [2], Ajay K. Banga [1,*]

[1] Center for Drug Delivery Research, Department of Pharmaceutical Sciences, College of Pharmacy, Mercer University, Atlanta, Georgia, 30341
[2] Central R&D Laboratory, New Product and Business Development Department, Kobayashi Pharmaceutical Co., Ltd., 1-30-3, Toyokawa, Ibaraki-city, Osaka, 567-0057, Japan

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ABSTRACT

Heparinoid is commonly used for the treatment of superficial thrombophlebitis, a condition wherein inflammation and clotting occurs in the veins below the skin surface. However, stratum corneum is a major barrier that limits the delivery of hydrophilic heparinoid, in and across the skin. The aim of the present study was to develop a nonirritant topical formulation for heparinoid incorporating chemical penetration enhancers and investigate the delivery of heparinoid across the human epidermis using *in vitro* vertical Franz diffusion cells. The developed oil-in-water nanoemulsions (NEs; NE-1 and NE-2) delivered higher amount of heparinoid (91.58 ± 25.75 μg/sq.cm and 62.67 ± 5.66 μg/sq.cm, respectively) after 72 h compared with the other developed formulations, which in turn also delivered significantly higher amount compared with commercial formulations: cream (1.78 ± 0.07 μg/sq.cm), ointment (9.95 ± 4.41 μg/sq.cm), and gel (0 μg/sq.cm) ($p$ <0.05). Transmission electron microscopy, polarizing light microscopy, and dynamic light scattering studies were performed to characterize the microstructure of these NEs with chemical enhancers. NE-1 was tested to be nonirritant with cell viability greater than 50% and a minimal release of IL-1α by using the "*in vitro* Epiderm tissue" model. Our results demonstrate that NE formulations represent a potential strategy for providing a localized therapy for the treatment of superficial thrombophlebitis.



## Introduction

Heparinoids are derivatives of heparin and exert a similar anticoagulant effect as heparin.[1] They block the blood clotting pathway by expediting the formation of antithrombin (AT) III, which inhibits both the formation and activity of thrombin.[2] Heparin is the most commonly used anticoagulants and is administered by injections for the treatment of venous thromboembolism.[3] Heparinoids are commonly used for the treatment of spontaneous or chemotherapy-induced superficial thrombophlebitis, a condition where inflammation and clotting are observed inside a vein below the skin surface.[4] They have also been used topically for the treatment of dry skin and asteatosis cutis[5,6] because they play an important role in regeneration of the skin tissue by stimulating the skin tissue proliferation.[7]

Parenteral administration is the conventional route for administering anticoagulants, but it has low patient compliance due to the invasiveness and the risk of complications associated with the fluctuations of heparin concentration in the blood.[8] In addition, it may not be advisable to use anticoagulants systemically for a relatively minor condition such as superficial thrombophlebitis. Thus, noninvasive and local delivery of anticoagulants would be preferable for the treatment of conditions occurring in close proximity to or on the skin. Transdermal or topical drug delivery offers an attractive alternative to injections through minimization of pain and possible sustained release of drugs.[9] The poor absorption of the large hydrophilic molecules across the stratum corneum is a major obstacle for effective delivery of heparinoid.

*Abbreviations used:* NE, nanoemulsion; PBS, phosphate-buffered saline; PG, propylene glycol; IPA, isopropyl alcohol; OA, oleic acid; NMP, N-methyl-2-pyrrolidone; IPM, isopropyl myristate.

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* *Correspondence to:* Ajay K. Banga (Telephone: 678-547-6243).
*E-mail address:* banga_ak@mercer.edu (A.K. Banga).

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In the present study, we hypothesized to develop a topical formulation for the effective delivery of heparinoid across the human epidermis. Strategies such as the incorporation of permeation enhancers or development of liposomes and nanoemulsions (NEs) have been widely used to enhance the percutaneous absorption in the skin.[10] Incorporation of chemical penetration enhancers is one of the most widely used approaches for improving topical and transdermal drug delivery as they are inexpensive and relatively simple to incorporate. Chemical permeation enhancers offer tremendous potential to enhance the transport of drug molecules by overcoming the barrier properties of the skin. They can promote the diffusion of an active substance across the skin barrier by partitioning themselves into the lipids bilayer, disrupting the lipid bilayer structure by extracting the lipids, and/or by interacting with the protein from the skin and thereby creating a pathway for drug permeation.[11] However, their efficacy in disrupting the skin barrier is limited at low concentrations, and they may result in irritation at high concentrations. Often, a mixture of these chemicals at lower concentration has been shown to provide high skin permeabilization as compared to individual chemicals at higher concentration, without causing skin irritation.[12] We hypothesized that the use of the skin permeation enhancers might increase the skin permeability of heparinoid, thus enhancing its delivery across the skin. To allow the incorporation of a useful amount of hydrophilic, lipophilic, or amphipathic chemical enhancers, NE was selected because of its high solubilizing potential.[13]

NEs are defined as colloidal dispersions that comprises oil phase, surfactant, cosurfactant, and aqueous phase; they may improve the delivery of active pharmaceutical ingredients into or across the skin. These consist of an optically isotropic and thermodynamically stable system with a droplet diameter usually within the range of 20-200 nm.[14] NEs offer advantages, such as long-term stability, ease of preparation, and considerable solubilizing capacity for a variety of drug molecules.[15] Moreover, incorporation of nonionic surfactants in NEs offer lower toxicity and skin irritation potential.[16] Interestingly, nonionic surfactants have also been shown to act as permeation enhancers by inducing fluidization of the stratum corneum lipids.[17] Therefore, we hypothesized that developing a topical NE formulation using nonionic surfactants with the incorporation of chemical enhancers could enhance the delivery of heparinoid.

Our results showed that, among all the developed formulations, nanoemulsion-1 (NE-1) and nanoemulsion-2 (NE-2) showed the highest delivery of heparinoid across the human epidermis. The heparinoid delivered into and across the skin were quantified using a sensitive enzymatic assay. Some studies reported that dermal absorption of heparinoid through animal and human skin could be detected by using histochemical staining or radiolabeling techniques.[18,19] However, these methods are not preferred, because of inadequate specificity or safety issues.

In addition to drug delivery, it is important to ensure the safety of the topical formulation; hence, the skin irritation potential of NE topical formulations was investigated. One of the commonly used models for testing skin irritation is the "*in vitro* reconstructed Epiderm tissue" model. This model has been routinely used to test skin irritancy by the European Center for Validation of Alternative Methods. The amount of released IL-1α and the percentage tissue viability were used to evaluate the skin irritation potential for NE. Because skin irritation induces the activation of innate immunity, the amount of cytokines released can be correlated to the irritancy potential of the formulation. Skin irritation triggers the release of cytokines and chemokines, with IL-1α being one of the indicators. Thus, in the present study, IL-1α was used as a potential marker for skin irritation.

The aim of the present study was to develop novel NE-based formulations containing chemical enhancers for the dermal delivery of heparinoid and investigate their *in vitro* permeation and skin irritation potential. The developed topical formulations were also compared with conventional topical formulations including gel, cream, and ointment on the market.

## Materials and Methods

### *Materials*

Heparinoid (8-16 kDa, p*K*a ~6.5) was provided by Kobayashi Pharmaceutical Co., Ltd. (Osaka, Japan). Chromogenix Coatest Heparin kit was purchased from Diapharma Laboratories (West Chester, OH). Ammonium hydroxide, acetic acid, 96-well plates, Tween 80, Span 20, propylene glycol (PG), and isopropyl alcohol (IPA) were obtained from Thermo Fisher Scientific (Fair Lawn, NJ). Oleyl alcohol, N-methyl-2-pyrrolidone (NMP), triethanolamine, and isopropyl myristate (IPM) were purchased from Millipore Sigma (Burlington, MA). Phosphate-buffered saline (PBS) (pH 7.4) and ethanol were purchased from MedSupply Partners (Atlanta, GA). Oleic acid (OA) was a gift sample from Croda Inc. (Snaith, UK). Dermatomed human skin was obtained from New York Fire Fighters (New York City, NY) and deionized water (MQ res: 18.2 MΩcm, perm C: 7.4 μS/cm) was generated by Mili-Q Direct 8 Water Purification System (Millipore Sigma, Burlington, MA). *In vitro* EpiDerm™ skin irritation test (EPI-200-SIT) kit was purchased from MatTek Corporation (Ashland, MA). Human IL-1 alpha/IL-1F1, Quantikine ELISA kit was purchased from R&D Systems (Minneapolis, MN).

### *Methods*

#### *Solubility Studies*

The solubility of heparinoid (0.3% w/w) was tested in different solvents and solvent combinations with chemical permeation enhancers (IPA, PG, IPM, OA, NMP, oleyl alcohol, triethanolamine, and ethanol). To solubilize lipophilic chemical permeation enhancers in the aqueous base, solubility was increased by the addition of surfactants (Tween 80 and Span 20) using the shake-flask method.[20] The fixed amount of drug (0.3% w/w) was added to a fixed volume of each solvent or solvent mixture (10 mL) in different flasks, which were vortexed, stirred, and placed on a shaker at room temperature (25 ± 0.5°C) for 24 h. The solubility of the NE formulations was evaluated via observation of clarity and phase separation.

#### *Preparation of NE Formulations*

NEs were prepared by using high-pressure homogenization (Nano DeBEE; BEE International, South Easton, MA), a high-energy emulsification method. The formulation compositions are listed in Table 1. Different concentrations of Tween 80/Span 20 or only Tween 80 were used as emulsifying and charge-inducing agents. Surfactant and cosurfactant were added in either aqueous or oil phase based on the solubility. Heparinoid (0.3% w/w) was dissolved in the aqueous phase. Aqueous and oil phases were prepared separately. The oil phase was added dropwise into the aqueous phase under constant stirring for the formation of an emulsion, which was then subject to high-pressure homogenization at a pressure of 10,000 psi for 10 cycles to produce stable NEs. The pH of all the formulations was determined using pH meter (Mettler Toledo Seven Compact, Columbus, OH) and was adjusted to approximately 7.0 by using 10% ammonium hydroxide. Selection for the appropriate ratio of surfactants was done based on its emulsification ability to form a homogenous NE formulation.

**Table 1**
Microstructure and Composition of Nanoemulsion Formulations

| Nanoemulsion (NE) | Microstructure | Excipient (% w/w) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Tween 80 | Isopropyl Myristate | Oleyl Alcohol | Span 20 | Ethanol | Isopropyl Alcohol | N-Methyl-2-Pyrrolidone | Deionized Water[a] |
| NE-1 | Oil-in-water | 3 | 5 | – | 4.5 | – | 15 | – | 72.5 |
| NE-2 | Oil-in-water | 10 | 16 | 10 | – | 10 | – | 8 | 46 |

[a] Heparinoid was included in deionized water at a concentration of 0.3% w/w in the final formulation.

*Characterization of NEs*

*Droplet Size and Surface Charge.* Droplet size and size distribution of heparinoid NEs were determined by dynamic light scattering using Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). NEs were diluted to minimize scattering effects before each measurement using deionized water. Results were described as size in nm for droplet size and polydispersity index (PDI) for size distribution. A sample of 1 mL of diluted NE (1:10 dilution) was loaded in a cuvette and placed in a thermostatic chamber at 25°C for particle size measurement and PDI ($n = 3$). The surface charge (zeta potential) present was also measured using Zetasizer Nano ZS90 in triplicate. Samples were diluted 1:10 times with deionized water before measurement. The samples were then injected into a capillary cell for zeta potential measurement. Results of zeta potential were described in mV.

*Morphology and Microstructure of NEs.* The isotropic nature of the NEs was observed under polarized light microscopy (Leica DM750, Buffalo Grove, IL). A drop of NE sample was placed on a glass slide, covered with a coverslip, and observed under polarized light (20× magnification). A dye test was performed by adding a water-soluble dye, methylene blue, to the NE, and the stained NE sample was observed in the same way.

The structure and morphology of heparinoid NEs were studied further using transmission electron microscopy (Hitachi H-7500, k Grove Village, IL). The samples were diluted with deionized water, and the NEs were deposited onto a 200-mesh copper grid, coated with formvar and carbon (Electron Microscopy Sciences, Fort Washington, PA). Samples were further negatively stained with 1% phosphotungstic acid, at room temperature. Excess fluid was absorbed by Whatman filter paper after allowing the samples to stand still for 10 min. One drop of 1% methylamine tungstate was applied and allowed to dry for 5 min just before examination.

*Viscosity.* The Brookfield viscometer (Spindle LV-1, Model DV-E; Brookfield Engineering Laboratories, Inc., Middleborough, MA) was used to measure the viscosity of NEs. The NE samples were sheared at the rate of 30 rpm for NE-1 and 50 rpm for NE-2. Measurements ($n = 3$) were carried out when the reading was stable.

*Refractive Index.* Refractive index of NEs was measured in triplicate using refractometer (ATAGO, Tokyo, Japan). The instrument was equilibrated before measurement by using distilled water (0.3 mL), and the sample (0.3 mL) was then added into the testing well at room temperature.

*Stability of NEs.* NEs were subjected to thermodynamic and temperature stress studies ($n = 3$). Prepared NEs were tested for short-term stability by centrifuging at 13,000 rpm for 30 min, and changes in the phase separation were visually observed. Further, the NEs were stored in 20-mL vials at 2 temperatures, 4°C and 25°C, for 1 month. The stability of the NE formulations was evaluated via observation of clarity and phase separation and the monitoring of particle size for 4 weeks.

In Vitro *Permeation Study*

*Preparation of Skin Samples.* Dermatomed human skin was thawed at 37°C and cleaned using deionized water. Epidermis layer was separated using the heat separation technique.[21]

*Skin Resistance Evaluation.* Integrity of the epidermis was evaluated using an electrical setup consisting of a digital multimeter (34410A 6 ½ digit multimeter; Agilent Technologies, Santa Clara, CA) and waveform generator (Agilent 33220A, 20 MHz function/arbitrary waveform generator). Epidermis was mounted on vertical Franz diffusion cells, and PBS (10 mM, pH 7.4) was added to the receptor and the donor compartments. A silver chloride electrode was placed in the donor, and a silver electrode was placed in the receptor. Load resistor ($R_L$) was connected in a series with the skin, and the drop-in voltage across the complete circuit ($V_0$) and across the skin ($V_S$) was recorded as displayed on the multimeter. Skin resistance ($R_S$) was calculated using the formula:

$$R_S = V_S R_L / (V_0 - V_S)$$

where $R_L$ and $V_0$ were 100 KΩ and 100 mV, respectively.

*Delivery of Heparinoid Across Human Epidermis From Different Formulations: Aqueous Solutions, NEs, and Marketed Formulations (Cream, Gel, and Ointment).* Delivery of heparinoid through human epidermis was investigated by performing *in vitro* permeation studies using vertical static Franz diffusion cells (PermeGear, Hellertown, PA). All heparinoid formulations including NE-1, NE-2, and aqueous solution formulations (F3, F4, F5, F6) were compared with commercially available conventional formulations (cream, gel, and ointment) for delivery of heparinoid across the human epidermis ($n = 4$ for each group). The receptor compartment contained 5 mL of PBS maintained at 37°C using a water circulation jacket built around the receptor chambers to bring the skin temperature to 32°C; the effective diffusion area was 0.64 $cm^2$. Human epidermis was mounted on Franz diffusion cell assembly with the stratum corneum facing the donor compartment. Formulations (100 μL) were applied on the skin in the donor compartment. Aliquots (300 μL) were withdrawn from the receptor compartment at predetermined sampling periods (0, 6, 24, 48, and 72 h) after the application of the formulation, and the drawn volume was replaced with fresh PBS. Description of the groups used is provided in Table 2.

The samples were analyzed by Chromogenix Coatest® heparin assay kit after suitable dilutions. Results obtained from blank (drug-free) formulations were subtracted from those obtained for test formulations to account for any absorbance values from the skin or formulation components. The results of the permeation experiments were plotted as cumulative amount of the drug permeated versus time.

*Skin Extraction Studies.* After permeation studies, the skin samples were removed from the Franz diffusion cells. The skin surface was thoroughly cleaned by wiping the formulation with Kimwipes® followed by cleaning with Q-tips (2×) dipped in receptor buffer and

**Table 2**
Composition of the Heparinoid Formulations Containing Various Enhancers

| Excipient (% w/w) | Formulation | | | | | |
|---|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 (NE-1) | F6 (NE-2) |
| Phosphate-buffered saline[a] | 18 | 10 | 10 | 10 | – | – |
| Propylene glycol | 72 | 89.5 | 72 | 80 | – | – |
| Oleic acid | – | 0.5 | – | – | – | – |
| Isopropyl alcohol | 10 | - | 10 | 9.6 | 15 | – |
| Ethanol | – | – | – | – | – | 10 |
| N-methyl-2-pyrrolidone | – | – | 8 | – | – | 8 |
| Triethanolamine | – | – | – | 0.4 | – | – |
| Oleyl alcohol | – | – | – | – | – | 10 |
| Isopropyl myristate | – | – | – | – | 5 | 16 |
| Tween 80 | – | – | – | – | 3 | 10 |
| Span 20 | – | – | – | – | 4.5 | – |
| Deionized water[a] | – | – | – | – | 72.5 | 46 |

[a] Heparinoid was included in phosphate-buffered saline or deionized water at a concentration of 0.3% w/w in the final formulation.

finally with dry Q-tips (2×). Skin extraction was then performed to determine the amount of the heparinoid in the skin. The skin exposed to the diffusion area was minced into small pieces using scissors and placed in 20-mL glass vials for extraction with PBS. The samples were kept on a roller shaker (New Brunswick Scientific Company, Inc., Edison, NJ) at room temperature (25°C) overnight at 150 rpm. Supernatant was filtered using a 0.45-μm filter and was then analyzed.

*Analytical Methods.* Heparinoid activity was measured using Chromogenic Coatest™ heparin test kits. The method was modified based on the manufacturer's protocol. The assay is based on the detection of anti-Xa activity generated by heparinoid present in the sample. Heparinoid formed a complex with AT. Factor Xa was added to neutralize the proportion of the amount of (heparinoid·AT) complex. The residual amount of factor Xa hydrolyzes the chromogenic substrate S-2222™, thus liberating the chromophore, pNA. The release of pNA (yellow color) is inversely proportional to the amount of heparinoid in the sample and is read photometrically at 405 nm. Linearity of the standard curves was determined over a range of 0.25-25 μg/mL with an $r^2 \geq 0.999$. Standards of heparinoid were prepared in receptor media for *in vitro* permeation studies. Twenty-five microliter of samples and standards were mixed with 25 μL of AT. The mixture was incubated at 37°C for 5 min. After the incubation, 25 μL of Factor Xa (prewarmed at 37°C) was added, and the mixture was incubated again for 30 s. Fifty microliters of S-2222 (prewarmed at 37°C) was added to the mixture and was incubated at 37°C for 4.5 min. The reaction was stopped by adding 75 μL of 20% acetic acid, and the optical density was read at 405 nm using a Synergy HT Microplate Reader (BioTek Instruments, Winooski, VT).

*Skin Irritation Test.* The NE-1 formulation was tested for skin irritation using *in vitro* 3D tissue culture Epiderm model in triplicate. Tissues were preincubated and then exposed to 30 μL of NE-1, negative control (NC), and positive control (PC). The PC was 5% sodium dodecyl sulfate, and the sterile Dulbecco's phosphate-buffered saline (pH 7.4) was taken as NC. Tissues were exposed to the formulation for 60 min, at 32°C, 5% $CO_2$ ($n = 3$ for each group), rinsed with Dulbecco's phosphate-buffered saline and replaced with 0.9 mL of fresh media. The media was collected for the cytokine analysis after incubation for 24 ± 2 h. Tissues were again incubated for 18 ± 2 h after the replacement of 0.9 mL of fresh media. After the completion of 42 h, cytotoxicity or irritation of the test materials was analyzed using methyl thiazolyl tetrazolium (MTT) assay according to the manufacturer's instruction. Tissues were exposed to MTT solution (1 mg/mL) and incubated for 3 h ± 5 min. After incubation, MTT was converted to blue formazan salt, which was extracted with 2 mL of isopropanol. The optical density was read at 570 nm using a Synergy HT Microplate Reader. Mean relative tissue viability of less than 50% was classified as an indication for irritant. Levels of IL-1α were further analyzed using quantikine ELISA kit according to the manufacturer's instruction. The data were plotted using a 4-parameter logistic curve fit.

*Data Analysis.* All the results are reported as mean ± SE unless otherwise specified. Statistical analysis was carried out using 1-way ANOVA or student's *t*-test. Results were quoted as significant where $p < 0.05$.

## Results and Discussion

### *Solubility Studies*

To match the drug content in the marketed products, the solubility of heparinoid at 0.3% w/w was investigated in single solvents and various solvent combinations along with several permeation enhancers. All the excipients and concentrations used strictly followed the Japanese inactive ingredient list.

The permeation enhancers included in the tests were OA, oleyl alcohol, ethanol, NMP, triethanolamine, IPM, IPA, and PG. Heparinoid (0.3% w/w) was found to be completely soluble in deionized water, PBS and PG, while not in ethanol. Heparinoid (0.3% w/w) was also found to be soluble in IPA, NMP, OA, and triethanolamine, but in combination with PBS and PG. Furthermore, Table 2 shows the compositions of the tested combinations of excipients. The proper ratio of solvents to achieve the target solubility was titrated, and all the formulations were tested for drug permeation across the human epidermis, as they did not show any visible phase separation or drug precipitation. Formulations F1-F4 were simple solutions, and F5 and F6 were NEs.

Disruption of the stratum corneum by permeation enhancers may enhance the delivery of heparinoid.[22] PG was added at a maximized ratio varying from 50% to 80% as it is nontoxic, compatible, and has high solubilizing effect on drugs.[23] It was used as both cosolvent and penetration enhancers.

Furthermore, as the drug was only soluble in the presence of PBS/water and to solubilize lipophilic chemical enhancers, NEs were developed using nonionic surfactants, Tween 80 and Span 20. IPM alone or together with oleyl alcohol was successfully incorporated in NEs as the oil phase. IPM and oleyl alcohol are widely used in dermal and transdermal delivery and are considered as effective penetration enhancers.[24] Nonionic surfactants such as Tween 80 and Span 20 were chosen as they are considered nontoxic and have low irritation potential. The effect of different concentrations of Tween 80 and Span 20 was investigated with respect to changes in size, particle size distribution (PDI), and physical stability. IPA and ethanol were incorporated in NEs in the aqueous phase (external phase) as cosurfactants.

### *Preparation of NE Formulations*

The compositions of heparinoid NE formulations are summarized in Table 2. A stable and translucent NE was formed when the surfactant (Tween 80) and cosurfactant (IPA and ethanol) ratio was 1:5 for NE-1 (F5) and 1:1 for NE-2 (F6), prepared by high-pressure homogenization technique. The NEs formed at these ratios were stable and isotropic. All the surfactants, cosurfactants, and permeation enhancers studied were pharmaceutically acceptable ingredients and were used at a physiologically acceptable concentration.

The rationale for selecting nanoemulsion for this study was to incorporate various lipophilic penetration enhancers. However, as

the drug is hydrophilic in nature, surfactants such as Tween and Span were added to formulate oil-in-water nanoemulsions. Thus, nanoemulsion enabled the incorporation of both lipophilic penetration enhancers and hydrophilic heparinoid. Most likely, these incorporated enhancers act on the stratum corneum, causing delipidization and dekeratinization of the membrane and thus assisting in permeation of heparinoid.

*Characterization of NEs*

The results of our study showed the development of successful NE formulations of heparinoid with optimum characteristics. Both NEs were stable and isotropic in nature. The pH of both the formulations was adjusted to pH 7.0.

*Droplet Size and Surface Charge*

The size distribution of NE droplets was determined by dynamic light scattering. The average droplet size for both the NEs was found to be < 200 nm; NE-1 (165.33 ± 0.27 nm) and NE-2 (198.07 ± 2.62 nm). This small diameter of both formulations could be attributed to the effect of cosurfactant molecules that lower the fluidity and surface tension of interfacial film, thereby decreasing the radius of nanodroplets.[25] The PDI value determines the homogeneity of droplet size. PDI for both NEs was found to be around 0.2 or less, which indicated a narrow size distribution. There was a decrease in the droplet size with an increase in the water content of NEs. Smaller droplet size resulted in a larger surface area of droplets, which possibly increased the interaction of NE with stratum corneum and potentially facilitated the percutaneous permeation.

NEs were also observed for other physiological properties such as surface charge. Zeta potential values provide information on the repulsive forces between particles in the emulsion system. A higher negative charge is known to prevent droplet coalescence during the formulation development.[26] Zeta potential of the NE formulations was −30.5 ± 0.3 and −15.8 ± 0.4 mV, for NE-1 and NE-2, respectively. Negative charge results in minimum aggregation, and pH within the physiological range is preferred for topical formulations.

*Morphology and Microstructure of NEs*

Polarized light microscopy also showed that the NEs were homogeneous, and the morphology of the NEs was further characterized using transmission electron microscopy. The droplets in the NEs appeared bright against a dark background. In general, majority of the NE droplets were spherical in shape without any visible agglomeration.

*Viscosity*

Viscosity of the NE is dependent on the surfactant, water, oil components, and their concentrations. Viscosity of the NEs was observed to decrease with the increase in water content; the viscosity of the NE-1 (17.8 ± 1.51 cps) was lower than that of NE-2 (79.1 ± 1.09 cps) (Table 3).

*Refractive Index*

Refractive index (RI) of the NEs represents the net RI of all the components of NEs; it indicated the isotropic nature of the formulation. The mean RI for NE-1 and NE-2 was 10.1 ± 0.1 and 28.2 ± 0.2, respectively. The lower RI for NE-1 might be attributed to a decrease in the water content, as water has a comparatively lower RI (RI of water is 1.334).

*Thermodynamic Stability and Effect of Temperature on Long-Term Stability of NEs*

Stress testing is required to assess the stability of the formulations. Oil-in-water NEs were subjected to the testing conditions such as centrifugation and storage under different temperatures. Centrifugation at 13,000 rpm for 30 min did not reveal any signs of phase separation or drug precipitation, indicating physical stability of NEs. Thermodynamic stability confers long shelf life to NEs. It helps to predict whether the emulsions will have kinetic stability or there will be phase separation.

Furthermore, stability of both NE formulations was tested at 2 different temperatures, 4°C and 25°C, by assessing the effect of these temperatures on the size of the droplets over a period of 4 weeks (Fig. 1). At the end of 4 weeks, both the NEs were stable with no changes in pH and characteristics such as clarity and color, or particle size, thereby suggesting that the prepared formulations were stable. The mean particle size for both the NEs was evaluated on the day of formulation (day 0), and the particle size was found to remain <200 nm for both NEs at 4°C. There was an increase in the particle size for NE-1 from 165.33 ± 0.27 nm to 176.0 ± 0.97 nm, and for NE-2, from 198.07 ± 2.62 nm to 336.93 ± 5.56 nm after 4 weeks. However, at 25°C, the droplet size remained approximately the same after 4 weeks: 165.33 ± 0.27 nm to 169.73 ± 0.69 nm (NE-1) and 198.07 ± 2.62 nm to 197.80 ± 2.30 nm (NE-2). Thus, both the NEs were found to be more stable at 25°C as compared with 4°C for 4 weeks. Therefore, room temperature could be considered more stable than 4°C for higher shelf life of these NEs.

In Vitro *Permeation Study*

*Delivery of Heparinoid Across Human Epidermis From Different Formulations: NEs, Solutions, and the Marketed Formulations (Cream, Gel, and Ointment)*

There was no passive permeation of heparinoid from its solution in PBS across human epidermis even after 72 h. However, all the formulations (F1-F6) containing permeation enhancers could deliver heparinoid across the human epidermis to the receptor compartment. The permeation of heparinoid across the skin was depicted in Fig. 2a.

Permeation of heparinoid from NE-1 (91.58 ± 25.75 μg/sq.cm) and NE-2 (62.67 ± 5.66 μg/sq.cm) after 72 h was significantly higher than the marketed formulations: cream (1.78 ± 0.07 μg/sq.cm), ointment (9.95 ± 4.41 μg/sq.cm), and gel (0 μg/sq.cm) ($p$ <0.05). However, there was no significant difference in the delivery of the heparinoid between both the NEs. The results obtained are consistent with previous studies in the literature that have shown the effectiveness of using NE for dermal delivery of drugs.[27] The range of values of the skin resistance observed in this study was similar with those reported previously.[28] A skin resistance of ≥10 kΩ ensures the integrity of the skin barrier.[29]

The higher permeation of heparinoid observed in the present study could be explained by the presence of permeation enhancers such as IPA and IPM in NE-1 and ethanol, NMP, oleyl alcohol, and IPM in NE-2. These chemical permeation enhancers affect the

**Table 3**
Physicochemical Parameters of Heparinoid Nanoemulsion Formulations

| Nanoemulsion | Droplet Size (nm) | Polydispersity Index | Charge (mV) | pH | Refractive Index | Viscosity (cps) |
|---|---|---|---|---|---|---|
| NE-1 | 165.33 ± 0.3 | 0.2 | −30.5 ± 0.3 | 7.0 | 10.1 ± 0.1 | 17.8 ± 1.5 |
| NE-2 | 198.07 ± 2.6 | 0.2 | −15.8 ± 0.4 | 7.0 | 28.2 ± 0.2 | 79.1 ± 1.1 |

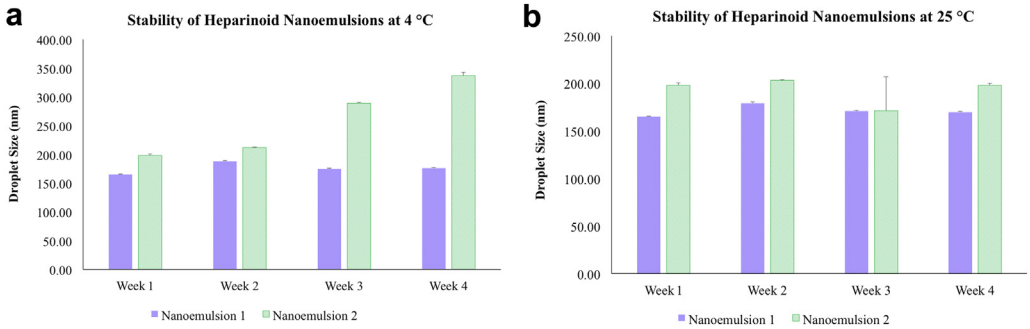


**Figure 1.** The stability of NEs; NE-1 and NE-2 at (a) 4°C and (b) 25°C for a period of 4 weeks.

intercellular lipids of the stratum corneum, which is believed to be the critical part of mechanism of action of penetration enhancement, thus altering the resistance of the stratum corneum toward heparinoid permeation.[30] Ethanol and IPA used in our study as permeation enhancers have been shown to enhance the skin permeation by a variety of mechanisms such as extraction of lipids and proteins that results in improved drug partitioning into the skin.[31] NE-2 had a combination of 4 permeation enhancers. Two permeation enhancers, oleyl alcohol (10%) and IPM (16%) were in the oil phase, and ethanol (10%) and NMP (8%) were in the aqueous phase. In comparison, NE-1 had only 2 enhancers IPM (5%) and IPA (15%) but showed slightly higher permeation (91.58 ± 25.75 μg/sq.cm) as compared with NE-2 (62.67 ± 5.66 μg/sq.cm). This result was consistent with our previous study, which reported that the drug permeation increased with an increase in water content of NE systems.[32] The permeation enhancement may be due to the increase in diffusion of the drug in NE-1, which had high water content (72.5%), as compared with NE-2 (46%).

Both NE formulations showed more than 16-fold, 4-fold, and 3-fold increase in the delivery of heparinoid compared with gel, cream, and ointment, respectively. This increase might be attributed to the synergistic action of permeation enhancers present in both NEs. The other formulations F2, F3, and F4 having OA (0.5%), NMP (8%), and triethanolamine (0.4%) also showed higher delivery of heparinoid 27.20 ± 21.52 μg/sq.cm, 41.65 ± 17.49 μg/sq.cm, and 62.40 ± 32.50 μg/sq.cm, respectively, as compared with the marketed formulations: cream (1.78 ± 0.07 μg/sq.cm), ointment (9.95 ± 4.41 μg/sq.cm), and gel (0 μg/sq.cm).

It was observed that both the NE formulations delivered a greater amount of drug across the epidermis compared with other formulations. This outcome is desirable as the target for delivery of heparinoid is the dermis, and here the amount present in the receptor compartment represented the delivery in the dermis. The amount of heparinoid delivered in the dermis by NE formulations was found to be higher than commercially available conventional formulations, including gel, cream, and ointment.

*Delivery of Heparinoid in Human Epidermis From Different Formulations: NEs, Solutions, and the Marketed Formulations: Cream, Gel, and Ointment)*

All the topical formulations developed were investigated for the permeation of heparinoid in and across the human epidermis. These formulations were also compared with the commercially available formulations such as cream, gel, and ointment. The drug-free groups were included in the *in vitro* permeation study to test the presence of endogenous heparinoid in the human epidermis. The results showed that no endogenous heparinoid was present in the human epidermis after 72 h of leaching into the receptor chamber of the Franz diffusion cell.

Delivery of heparinoid (0.3% w/w) from different formulations in the human epidermis is depicted in Figure 2b. Although all formulations could deliver heparinoid in the epidermis, the gel formulation showed the highest delivery (95.82 ± 2.33 μg/sq.cm). The permeation enhancer NMP showed significantly ($p$ <0.05) higher delivery (9.73 ± 1.53 μg/sq.cm) in the skin, compared with cream (2.56 ± 0.66 μg/sq.cm) and ointment (2.01 ± 0.30 μg/sq.cm).

It should be noted that the skin retention of heparinoid from both NE formulations was lower as compared with gel and other aqueous formulations. Heparinoid retention from NE-1 and NE-2 was found to be 1.28 ± 0.4 and 3.28 ± 1.1 μg/sq.cm, respectively, which was significantly lower than that from Attonon gel formulation. However, the amount of heparinoid was similar and not significantly different between NE-1 and NE-2, when compared with conventional commercial cream and ointment formulation of

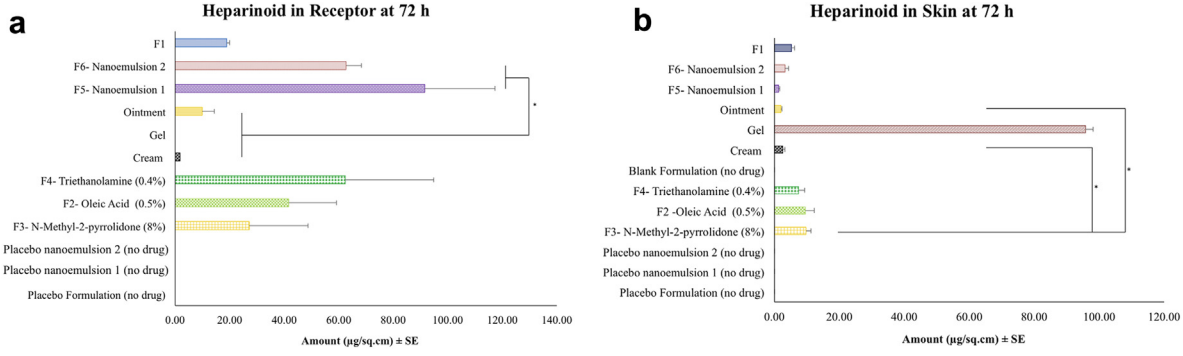


**Figure 2.** (a) Permeation of heparinoid in receptor compartment ($n$ = 4, mean ± SE; *$p$ <0.05 for NE-1 and NE-2 vs. gel, cream, and ointment); (b) permeation of heparinoid in the skin ($n$ = 4, mean ± SE; *$p$ <0.05 for F3 vs. cream and ointment).

heparinoid. No heparinoid was detected in the skin after treatment with placebo formulations of NEs. Other solution formulations, F2 (9.73 ± 1.5 μg/sq.cm), F3 (9.50 ± 2.8 μg/sq.cm), and F4 (7.43 ± 1.9 μg/sq.cm), showed higher amount of heparinoid in the skin compared with cream (2.56 ± 0.7 μg/sq.cm) and ointment (2.01 ± 0.30 μg/sq.cm). The amount of heparinoid retained in the skin was the highest for gel (95.82 ± 2.3 μg/sq.cm). The high skin retention from gel could be attributed to the retention of the drug in the skin. However, because the target site of delivery for its therapeutic action is on dermis, the delivery of heparinoid in the epidermis was of lesser importance.[6]

*Skin Irritation Test for the NE-Based Formulation*

An important consideration related to the NE-based delivery systems is the toxicity of its components because large amounts of surfactants may cause skin irritation when applied topically. Thus, selection of surfactants at a safe concentration is necessary. It is, therefore, important to determine the appropriate surfactant concentration, preferably the minimum concentration that can be used in the formulation. Nonionic surfactants are relatively nontoxic than their ionic counterparts.[33] Thus, for our study, we incorporated nonionic surfactants such as Tween 80 and Span 20.

The relative percentage viability of Epiderm™ tissues treated with PC, NC, and NE-1 formulation is shown in Figure 3. Any substance is considered as an irritant if the percentage of tissue viability is less than 50%. Results of the MTT assay (mean ± SD) showed that the tissue viability for the NC was 100 ± 5.7%, whereas the PC- and NE-1-treated groups showed tissue viability of 3.3 ± 0.1% and 75.9 ± 17.8%, respectively. Viable tissues convert MTT into a blue formazan salt formed by cellular mitochondria, and the amount of the converted product is proportional to the viability of the tissue. As the viability of NE-1-treated tissue was more than 50%, it was predicted to be nonirritant.

A 3D cell culture was treated with NE-1 formulation. After 24 h of exposure, cytokine analysis (mean ± SD) was performed with Quantikine ELISA kit. Irritation to the skin may lead to elevated cytokine levels. IL-1α was chosen as an end point to determine the efficacy in this study. The basal level of cytokine for the untreated skin (NC) was found to be 28.48 ± 6.9 pg/mL. Treatment with NE-1 did not increase IL-1α levels (32.59 ± 5.9 pg/mL) (Fig. 4), which were significantly lower than the PC ($p$ <0.001). The PC group (5% SDS) was considered as an irritant and had a basal level of 203.97 ± 40.0 pg/mL of IL-1α.

The study clearly has illustrated the impact of permeation enhancers in the formulation for the delivery of heparinoid. The NE formulation, NE-1, was further confirmed to be nonirritant, and thus shows potential to be used as a safe and effective formulation for the topical delivery of heparinoid.

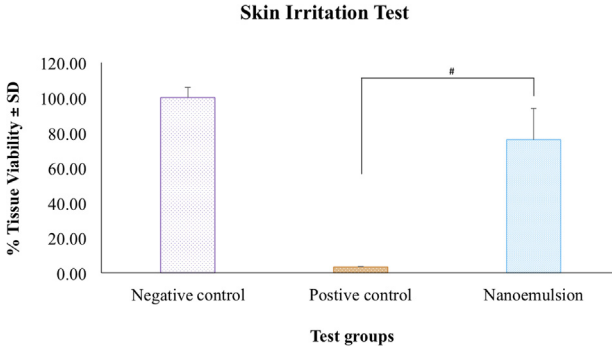


**Figure 3.** The relative tissue viability obtained from the skin irritation test *in vitro*. Tissues were exposed to 5% SDS (positive control) and NE-1 formulation. ($n$ = 3, mean ± SD; #$p$ <0.001, NE-1 vs. positive control). Untreated tissue was used as the negative control. Values are expressed as mean % tissue viability.

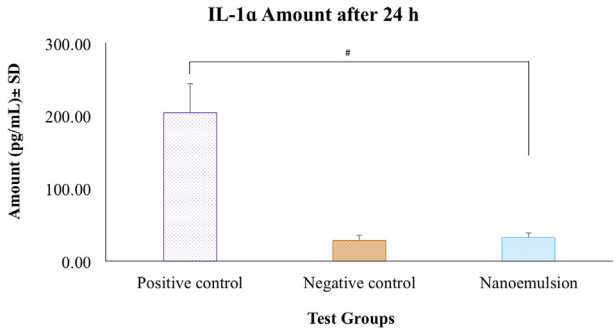


**Figure 4.** Amount of IL-1α released from human epidermal tissues. Tissues were exposed to 5% SDS (positive control) and NE-1 formulation ($n$ = 3, mean ± SD; #$p$ < 0.001, NE-1 vs. positive control). Untreated tissue was used as the negative control.

## Conclusion

An appropriate composition of NE formulations including the type and level of oil phase, surfactant, cosurfactant, aqueous phase, and permeation enhancers is important to achieve delivery into or across the skin. In the present study, we developed and characterized oil-in-water NE formulations for the dermal delivery of heparinoid. *In vitro* permeation studies showed that the application of both the NE formulations resulted in significantly enhanced topical delivery of heparinoid across epidermis as compared with other conventional topical formulations (cream, gel, and ointment) ($p$ <0.05). Furthermore, the penetration of heparinoid was found to increase with the use of oleyl alcohol and IPM as permeation enhancers as compared with other enhancers. In addition to enhanced permeation, MTT assay showed the dermal safety of NE formulation. In conclusion, NE formulations can be promising vehicles for topical delivery of heparinoid and a potential alternative to invasive techniques such as injections for the delivery of heparinoid across the skin.

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