Development of Lecithin/Chitosan Nanoparticles for Promoting Topical Delivery of Propranolol Hydrochloride: Design, Optimization and In-Vivo Evaluation

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Abstract
Propranolol (PPL) administered orally is considered as the first line drug for the treatment of infantile hemangioma, however several systemic adverse effects limit its use. For this reason, our work tackles the development and evaluation of PPL loaded chitosan nanoparticles (NPs), as an effective alternative for the treatment of infantile hemangioma. PPL-NPs were prepared using the double emulsion technique and the influence of the formulation variables on drug entrapment efficiency (EE), particle size (PS), percent released after 24 h (%R24h) and zeta potential (ZP) were optimized using full factorial design. Two systems, namely F3 and F28 showing highest EE, ZP and %R24h with lowest PS, were fully characterized for DSC and TEM and incorporated into hydrogel with adequate viscosity. After ensuring safety for the selected nanoparticle, the hydrogel containing the optimized system was applied topically to rats. The in-vivo skin deposition in rats showed an accumulation of propranolol from the lecithin/chitosan nanocarrier by 1.56–1.91-fold when compared to the drug solution. The obtained result was further supported by the confocal laser scanning microscopy which showed fluorescence across the skin. PPL-HCL-loaded lecithin/chitosan nanoparticles could be considered as a potential candidate for treating infantile hemangiomas (IH) by maintaining therapeutic concentration topically while minimizing systemic side effects.

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Introduction
Propranolol (PPL), a β blocker used for cardiovascular disorders, has proved effective in treating infantile hemangioma (IH).1 The French Incorporation, Pierre Fabre Pharmaceuticals repurposed propranolol for the treatment of proliferating IH in the form of oral solution, following an international Phase 3 clinical study and the FDA approval in 2014. Encouraged by the success of oral propranolol, pediatricians have applied β blockers, including PPL, topically and have reported on their efficacy in treating superficial forms of IH. Since there are no marketed forms of topical PPL, clinicians administered ointments and gels loaded with PPL for topical use in order to minimize the adverse effects of oral β blockers.

Chen et al., 2015 reported an 86% response in patients treated with topical 0.5% nano-propranolol hydrogel.2 Also, Xu et al., proved that of 25 children with IH treated with 1% propranolol ointment, 90% showed either good or partial responses to the topical treatment.3

In a study by Mouhari-Toure et al., a patient with IH showed fast regression of IH following a treatment with 2% propranolol ointment.4 Several other authors studied this subject and proposed a possible mechanism of the action of the β blocker on IH.5,6 Zimmermann et al., provided an extensive review on the use of PPL for hemangioma treatment.5,7

Although the exact mechanisms by which it acts are not clearly defined, applying PPL topically without knowledge of the amounts of drug deposited and or accumulated in the skin as well as the amount of drug permeating through the skin to enter the circulation, requires a topical delivery system where these attributes are designed in the product and can be easily controlled.

Nanoparticles have advantages as topical carriers because of their unique characteristics. Being of small particle size they can...
increase substantially the contact area with the skin's stratum corneum (SC) and enable the encapsulated drug to penetrate the skin. This is an important factor for topical application, i.e. it enables the particles to reside selectively in the different layers of the skin (SC, epidermis, dermis).

Nanovesicles prepared from lecithin and chitosan have been largely studied and have been reported to influence the flux, to enhance drug deposition and accumulation of drugs in rat skin and even to control the permeation through the skin. Sonvico et al., demonstrated that positively charged lecithin/chitosan nanovesicles represented good topical delivery platform capable of attaching to negatively charged skin.8 Also, Özcan et al., developed a lecithin/chitosan formulation as topical carrier of diflucortolone valerate to enhance its anti-inflammatory activity. Authors proved that the lecithin/chitosan combination of the nanoparticles imparted sustained release of the drug and enhanced the edema inhibition compared to the commercial product. The nanoparticles were shown to accumulate in the SC and the epidermis without permeation. The skin retention was better in comparison with the commercial cream.9

Tan et al., studied the in vitro and the in vivo drug deposition of quercetin from lecithin/chitosan nanoparticles. They found that the prepared nanoparticles enhanced the amounts of quercetin remaining in the dermis and epidermis when compared to the drug solution. The amount of drug permeating the skin was greatly reduced during the 12 h ex vivo experiment. The authors assumed that the difference could be due to the properties of the nanoparticles, i.e. the lipophilic character of lecithin, the positive charge of chitosan combined with their large surface area. They suggested that the structure of the lecithin/chitosan nanoparticles could influence the retention of the particles within the skin layers and thus enhance the therapeutic effects of quercetin as antioxidant and anti-inflammatory drug when applied topically.10

The aim of this study is to develop an effective topical delivery system for PPL-HCL to be recommended for topical treatment of mild forms of IH. The goal is to tailor the carrier system to be able to retain adequate drug concentrations in the skin's epidermis and or dermis. Targeting of the drug topically offers the advantages of reducing the drug dose along with decreasing systemic toxicity associated with oral drug administration.

Materials and Methods

Materials

Propranolol hydrochloride (PPL-HCL) was purchased from El-Nasr Pharmaceutical Company, Cairo, Egypt. Lipoid S45 (lecithin) was a gift from Lipoid GmbH, Switzerland. Glycerol monostearate (GMS), poloxamer 188, Tween 80, low molecular weight chitosan (50,000–190,000 Da) and cellulose membrane (molecular weight cutoff 12,000–14,000) were purchased from Sigma Chemical Company, St. Louis, USA. All other reagents were of analytical grade supplied by El-Nasr Company for Pharmaceutical Chemicals, Cairo, Egypt.

Methods

Preparation of PPL-HCL Nanoparticles

PPL-HCL loaded nanoparticles were prepared by the previously reported w/o/w type double emulsification method.11 PPL-HCL was dissolved in 1 ml distilled water containing 0.125% w/v Tween 80. Lipid matrix was prepared as ratio 1:1 GMS and Lipoid S45 dissolved in 5 ml dichloromethane (DCM).12 The lipid matrix was added slowly to the aqueous solution and homogenized for 2 min at 22,000 rpm to produce white cloudy primary emulsion. The resultant primary emulsion was poured on 100 ml distilled water containing poloxamer 188 (P188) and chitosan13 and homogenized for additional 5 or 10 min at 22,000 rpm. The organic solvent was removed under vacuum at 40 °C by a rotary evaporator (Rotavapor, Buchi-M/HB-140, Switzerland). The resulting PPL-HCL loaded nanoparticles were allowed to cool to room temperature, and then stored in the refrigerator for further investigations. The detailed description of different formulations composition is illustrated in Table 1.

Characterization of PPL-HCL Nanoparticles

Determination of Entrapment Efficiency, EE%. The percentage of drug incorporated in the prepared nanoparticles was calculated after separation of the nanoparticles by centrifugation. Five milliliter (5 ml) of the investigated nanoparticle was centrifuged at 9000 rpm with temperature maintained at −4 °C for 90 min using a cooling centrifuge (Union 32R, Hanil Science Industrial, Gimp-și, Gyeonggi-do, Republic of Korea). The supernatant was collected, filtered through Millipore membrane filter (0.22 μm) then diluted with distilled water and measured spectrophotometrically11,14 at a wavelength of 289 nm (Shimadzu UV-VIS double beam spectrophotometer (2401/PC), Japan). The amount of incorporated drug was determined from the difference between the initial drug content and the free drug found in the supernatant using the following equation15,16:

\[
EE\% = \frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}} \times 100
\]

Measurement of Particle Size, PS. The PS of the prepared nanoparticles was measured by photon correlation spectroscopy, using a Zetasizer Nano ZS (Malvern Instrument, Worcestershire, UK) at a fixed angle of 90° at 25 °C. Samples were diluted appropriately with distilled water before measurement. The measurements were conducted in triplicates.

Measurement of Zeta Potential (ζ). The surface charge of the prepared nanoparticles was determined by the measurement of zeta potential (ζ) with photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instrument, Worcestershire, UK). About 1 ml of the nanoparticle dispersion was diluted to 10 ml with distilled water. After shaking, the suspension was transferred into a standard cuvette for zeta potential measurement. The sample temperature was maintained at 25 °C and measurements were conducted in triplicates. The zeta potential values were calculated according to Smolochowski’s equation17:

\[
\mu = \frac{\xi \epsilon}{\eta}
\]

Table 1

Factors and Levels of 2^5 Full Factorial Design for PPL-HCL Nanoparticles.

<table>
<thead>
<tr>
<th>Independent Variables (Factors)</th>
<th>Factors Levels</th>
</tr>
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<tbody>
<tr>
<td>X1: Drug Amount (mg)</td>
<td>40  80</td>
</tr>
<tr>
<td>X2: Poloxamer 188 (3w/v)</td>
<td>2   4</td>
</tr>
<tr>
<td>X3: Lipid/Lecithin (1:1,w/w) (mg)</td>
<td>200 400</td>
</tr>
<tr>
<td>X4: Chitosan (3w/v)</td>
<td>0.1  0.2</td>
</tr>
<tr>
<td>X5: Homogenization time (min)</td>
<td>5   10</td>
</tr>
</tbody>
</table>

Dependent Variables (Responses) | Constrains |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1: Entrapment Efficiency (EE %)</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y4: Particle Size, PS (nm)</td>
<td>Minimize</td>
</tr>
<tr>
<td>Y2: Percent Released (%R_{24})</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y5: Zeta Potential</td>
<td>Maximize</td>
</tr>
</tbody>
</table>
Where \( \mu \) is the electrophoretic mobility, \( \zeta \) is the zeta potential, \( \epsilon \) is the dielectric constant and \( \eta \) is the viscosity.

**In-Vitro Drug Release Studies**

The release of free PPL-HCL and PPL-HCL from nanoparticles were determined by the dialysis bag diffusion method using phosphate buffer pH 5.5 as dissolution medium. The sample was placed into a cellulose acetate dialysis bag (molecular weight cutoff 12,000–14,000) and sealed at both ends. The dialysis bag (donor compartment) was immersed in the receptor compartment containing 50 ml of dissolution medium, stirred at 100 rpm, and maintained at 32 ± 0.5 °C. About 2 ml sample was withdrawn from the receptor compartment and same volume of fresh release medium was added to maintain sink conditions at fixed time intervals (0.15, 0.5, 1, 2, 3, 4, 5, 6, 8, and 24 h). The percentage of PPL–HCL released was measured spectrophotometrically at 289 nm. Each experiment was carried out in triplicate and the results were expressed as the mean values ± S.D. In addition to the release profiles, characterizing the release kinetics can help understand the mechanisms involved as part of the optimization process. For this reason the data were analyzed using linear regression equations, and the order of the drug released from the different formulations was recorded (zero order, first order or Higuchi diffusion model). Korsmeyer–Peppas model (log cumulative percentage drug release versus log time) was also taken in consideration in order to determine the mechanism of PPL–HCL release from NPS formulations.

**Optimization of PPL–HCL Nanoparticle Using Full Factorial Design**

Preliminary experiments were conducted to explore the ranges of the nanoparticle variables within which a satisfactory entrapment of the drug in the nanocarriers can be achieved. They included investigation of the effects of the lipid/lecithin ratio (1:1 and 1:2); the surfactant type (Poloxamer 188 and 407, Brij 78 and Cremophor) and the surfactant concentration (1% and 2%).

A 2\(^2\) full factorial design was used for the preparation of PPL–HCL loaded lecithin/chitosan nanoparticles utilizing Design-Expert® 7.0 software in order to investigate the effect of the selected formulation variables. In the present design, five formulation variables (referred to as “factors”, X) were selected at two levels to investigate their effects on the following properties (referred to as “responses”, Y): Entrapment Efficiency, EE%, Particle size, PS nm, in vitro percent released (% R\(_{24h}\)), and zeta potential. The different variables and responses are illustrated in Table 1.

To evaluate the significance of the influence of the variables on the responses, the data obtained from the factorial design were fitted to a generalized linear regression model and analyzed with the SPSS® software using one-way analysis of variance (ANOVA) followed by the least-significant difference test (LSD). The model terms describing the effects were considered statistically significant for \( p < 0.05 \).

**Characterization of Optimized Nanoparticles**

**Transmission Electron Microscopy, TEM.** The morphology of PPL-HCL loaded nanoparticles was examined by transmission electron microscopy (TEM) (Jedl JEM–1230, Tokyo, Japan) after diluting the nanoparticle suspensions with distilled water (1:10). A sample of the dispersion was placed on a carbon coated copper grid and left to adhere on the carbon substrate for about 1 min. A drop of 1% phosphotungstic acid solution was added to act as a negative staining agent and the solution in excess was removed by a tip of filter paper. After being stained, samples were left to dry at room temperature for 10 min before measurements at appropriate magnifications.

**Incorporation of PPL–HCL Loaded Nanoparticles Into Hydrogel**

To evaluate the nanoparticles for topical application, the optimized nanoparticles or systems were incorporated into hydrogel using Carbopol 940. Carbopol 940 (1% w/w) was added to the PPL–HCL loaded nanoparticles dispersion with continuous stirring till the formation of the gel. Tri-ethanolamine was added in order to neutralize the acidic nature of Carbopol 940.

**Characterization of PPL–HCL Loaded Nanoparticles Hydrogel pH Value Measurement.** The pH value of the hydrogels was determined using pH meter (Jenway, Bibby Scientific Limited, Staffordshire, UK) at 25 °C. The pH meter was previously standardized using a buffer solution at pH 7.0 and pH 10.0. Briefly, 0.5 g of PPL–HCL nanoparticles gel was diluted tenfold with bidistilled water and mixed well prior to measurement, and pH was recorded. Three measurements were carried out and the mean values ± S.D. were reported.

**Rheological Study.** The rheological measurements were performed at a temperature of 25 ± 0.1 °C using a computerized rheometer equipped with a cone and plate geometry (plate diameter 40 mm, cone angle 4°) (Anton Paar, Physica MCR 301, Germany). Each sample continuous variation of the speed rate from 1 to 100 s\(^{-1}\) then backward from 100 to 1 s\(^{-1}\) was applied, and the resulting viscosity was measured. The rheological properties of the samples were determined by plotting the viscosity versus the shear rate (viscosity curves) and the shear stress versus the shear rate (flow curves).

**Cytotoxicity Study of PPL–HCL Nanoparticle Containing Hydrogel**

When selecting the constituents of the nanoparticles, it is important to determine the viability of cells at varying concentrations of the carrier materials. For this reason, the cytotoxicity test is considered as one of the most important indicator of the biological evaluation of the system in vitro.

The cytotoxic activity of the PPL–HCL loaded nanoparticle hydrogels as well as free drug hydrogel was performed against human normal skin fibroblast (HFB4 cells). A 96 well tissue culture plate was inoculated with 1 × 10\(^5\) cells/ml (100 µl/well) and incubated at 37 °C for 24 h to develop a complete monolayer sheet. Growth medium was decanted from 96 well micro titer plate after confluent sheet of cells were formed, cell monolayer was washed twice with wash media. Two-fold dilutions of tested sample were made in RPMI medium with 2% serum (maintenance medium). 0.1 ml of each dilution was tested in different wells leaving 3 wells as control, receiving only maintenance medium. Plate was incubated at 37 °C and examined. Cells were checked for any physical signs of toxicity, e.g. partial or complete loss of the monolayer, rounding, shrinkage, or cell granularity. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution was prepared (5 mg/ml in PBS) (BIO BASIC CANADA INC). 20 µl MTT solution was added to each well and placed on a shaking table, 150 rpm for 5 min, to thoroughly mix the MTT into the media and incubated (37 °C, 5% CO\(_2\)) for 1–5 h to allow the MTT to be metabolized. Resuspended formazan (MTT metabolic product) in
200 μl DMSO was placed on a shaking table, 150 rpm for 5 min, to thoroughly mix the formazan into the solvent. The optical density (OD) was measured at 560 nm and subtracts background at 620 nm.\textsuperscript{21-23}

Irritation Test

Skin irritation test was conducted in order to evaluate and observe the skin irritation potential of the optimized hydrogels and the free drug on Wistar rats.\textsuperscript{24} Nine healthy rats were assigned in the study, where the rats had free access to food and water. The back of the rat was shaved carefully avoiding peripheral damage 24 h before conducting the study. About 0.05 ml of nanoparticles was applied on the left side of the hair free skin of rats by uniformly spreading, leaving the right side as control. The left side of the dorsal skin surface was observed for any visible change such as erythema (redness) after 24, 48 and 72 h after application. The irritation potential or erythema score was recorded depending on the degree of erythema observed: no erythema = 0, slight erythema (barely visible light pink) = 1, moderate erythema (dark pink) = 2, moderate to severe erythema (light red) = 3 and severe erythema (extreme redness) = 4.\textsuperscript{25}

In Vivo Skin Deposition Study

Experimental Animals. A total of 72 male Wistar rats, weighing 130–150 g, were used in the in vivo study. The animals were supplied with standard diet and tap water and placed individually in cages. The animal experiments were carried out in accordance with the regulatory guidelines approved by the National Research Centre Animal Care.

Skin Deposition Measurements. On the day of the experiment, the rats were randomly separated into 3 groups with 24 animals in each group. Bottle caps which served as drug pools with an area of 4.9 cm\textsuperscript{2} were fixed to the rat dorsal skin which was shaved with an electric clipper 24 h before application of the sample. 0.05 ml containing 1.7 mg/kg\textsuperscript{26} of drug of the selected PPL-HCL loaded nanoparticles gels or free PPL-HCL gel was added exclusively on the drug pool. The groups were treated according to the following pattern: Group I: topical application of free drug hydrogel. Group II: topical application of PPL-HCL - loaded nanoparticles hydrogel (formula F3). Group III: topical application of PPL-HCL - loaded nanoparticles hydrogel (Formula F28). At selected time intervals (1, 2, 4, 6, 8 and 24 h), four animals from each group were sacrificed by cervical dislocation and the treated skin area was excised, washed with saline solution then placed in 10% formalin. Vertical slices of 20 μm thickness were prepared using a microtome, and then subjected to normal and fluorescence light microscopy (20× magnifications, BX-8000, Keyence, Neu-Isenburg, Germany). The fluorescence was recovered by exciting the samples at 560 nm and setting the camera integration time to 1/45 s for RhB. The arbitrary pixel brightness values were evaluated using the image analysis software BZ Analyzer (Keyence, Neu-Isenburg, Germany) and the relative dye contents within the stratum corneum, viable epidermis and dermis, respectively, were quantified.

Effect of Storage on the Properties of the Optimized Systems

The effect of three months storage on the encapsulation efficiency and particle size of the optimized hydrogels was conducted at 4 ± 2 °C in screw-capped vials. Samples were taken periodically at intervals of 1 month for 3 months period and examined for encapsulation efficiency and particle size.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD) from triplicate measurements. Statistical analyses were conducted with the SPSS® software using one-way analysis of variance (ANOVA) followed by the least significant difference test (LSD). p value < 0.05 was set as a level of statistical significance.

Results and Discussion

Preparation and Characterization of Nanoparticles

The double emulsion solvent evaporation (DESE) method has been employed for the preparation of nanoparticles incorporating both hydrophilic and hydrophobic drug molecules. The use of DESE for preparation of nanoparticles and subsequent coating with chitosan without cross-linking was reported by Petkar et al., 2018; who employed this technique for improved pulmonary delivery of rifampicin. It has been also reported that by combining lecithin and chitosan, nanoparticles of spherical form can be obtained because of electrostatic interaction between negatively charged lecithin and positively charged chitosan. The chitosan layer with high positive charge surrounding the lipophilic core containing the entrapped drug molecules can thus act as protective coat stabilizing the nanostructure.\textsuperscript{31}

Literature search revealed no previous studies on the utilization of chitosan-based PPL-HCL nanoparticles for topical drug delivery. The feasibility of using DESE and chitosan was therefore a challenging investigation to develop a nanocarrier as potential platform for topical treatment of infantile hemangiomas.

Optimization of Formulation Using Full Factorial Design

Factorial designs are useful tools for quality design of systems which enable defining the design space for the desired product properties. They represent statistical analysis of experimental data obtained during the system development stages. The 2\textsuperscript{5} full factorial design of the five factors at two levels shown in Table 1, generated 32 possible combinations. The nanoparticles and the responses obtained are listed in Table 2.

Statistically significant values obtained by ANOVA were characterized by a p value less than 0.05. The estimates of the single factors have the terms, X1 … X5, whereas double terms were used to describe the interactions between the factors (e.g. X1*X2 ... X1*X4).
Effect of Formulation Variables on the Response Parameters

Entrapment Efficiency, EE % (Y1). The results showed that the EE% was affected positively by the single factors and negatively by the interaction X3*X5, all being statistically significant with the exception of the factor X2 (Poloxamer 188) which had a p value > 0.05. The largest estimate was due to the lipid/lecithin mixture (X3 = 5.24) signifying an increase in entrapment efficiency with the increase in the concentration of lipid mixture. The lipid/lecithin mixture in the 1:1 ratio used in the preparation of the nanoparticles is responsible for the formation of the lipid core entrapping the drug, which is of a hydrophilic nature and thus capable of locating in the core. It has been suggested that the presence of the lipid in the mixture can disturb the solid lipid structure making space available for the incorporation of the drug. This finding is in agreement with our findings, where increasing the amount of lipid more space can be created for the entrapment of the drug molecules.

Chitosan (a positively charged polymer) can bond with the negatively charged core containing lipid/lecithin mixture and in the process it helps sealing the core. As more chitosan becomes available, the sealing capacity increased resulting in increase in the EE%.

The estimate of the effect of drug load (X1) was also significant (X1 = 2.55, p < 0.05) indicating that EE% increased as more drug was available for incorporation. The effect of (X5) was with a positive sign (X5 = 1.97) indicating that the entrapment was more favorable at the longer homogenization time. On the other hand, the negative estimate of the interaction between the lipid/lecithin and the homogenization time means that the increase in EE% at the increased amount of lipid/lecithin becomes negatively affected as the homogenization time increased (i.e. the increase is steeper at 5 min compared to 10 min of homogenization time). This is illustrated graphically by the three dimensional response surface plot obtained when X3 and X5 are varied while the other factors are kept constant (Fig. 1a). It can be seen that the highest value of EE% was obtained with X3 at the higher level and X5 at the lower level. All remaining values of EE% were below this point. The goodness of fit to the regression model was satisfactory enough at R² = 0.85 and thus the linear equation for EE % (Y1) including the statistically significant estimates can be presented as:

\[
Y_1 = 46.61 + 2.55X_1 + 5.24X_3 + 2.21X_4 + 1.97X_5 - 1.92X_3X_5
\]

Where 46.61 is the Intercept value obtained from the model.

Particle Size PS nm (Y2). Table 2 showed that the PS values of all formulations were within the nanometer scale and ranged from 156.1 to 585.2 nm. The smallest sizes were observed at the upper level of X5 (homogenization time, 10 min) for F3 and F19, where the estimate of X5 has the highest negative value at p = 0.002 indicating a decrease in PS as X5 is increased from 5 to 10 min.

The estimates of X1 and X3 had positive values indicating an increase in PS as the drug load and the chitosan amount increased. Although the effect of X4 was statistically insignificant at p value of 0.56, it is important to note that the size increased after coating with chitosan. As the amount of chitosan increased, the lipiddchitosan interactions increased enough to fill the available space in the bilayer increasing the size of the vesicles.

The increase in drug load did not influence the PS in any significant way (p = 0.635). This is in agreement with other studies performed by Mahmoud et al., who found no difference in PS between curcumin loaded and drug free nanoparticles.

The estimate of the single factor X2 (Poloxamer 188) had significant influence, increasing the PS as its level increased.
Since Poloxamer 188 (a nonionic block linear copolymer) is capable of reducing surface tension and increasing miscibility, its effect on PS in the present study is ambiguous. On the other hand, due to its high molecular weight it could have increased the viscosity reducing the homogenization efficiency and thus increasing the PS.

The effect of the interaction X3*X4 on PS is best illustrated graphically with the response surface plot, where the two factors are varied simultaneously, while keeping the other variables at fixed values (Fig. 1b). It can be seen from the plot that the most favorable values for PS were obtained when both factors were at their lower levels.

The linear equation for PS (Y2) is written below as:

\[ Y2 = 310.58 + 49.67 X2 -71.23 X5 + 39.23 X3 \times X4 \]

with 310.58 as the Intercept.

Zeta Potential. The Zeta potential values obtained from the factorial design were positive and were retained generally above 40 (Table 2). The largest effect on the zeta potential was due to the chitosan, the remaining factors not exhibiting any significant influence on this response. High positive charges on nanoparticles

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**Fig. 1.** Response surface plots of (a) Entrapment Efficiency (EE%) illustrating the interaction X3*X5; (b) Particle Size (PS nm) illustrating the interaction X3*X4 and (c) Percent released (%R24) illustrating the interaction X1*X4.
prepared with addition of chitosan have been reported elsewhere.\textsuperscript{33} Highly positively charged chitosan-coated nanoparticles remain stable during storage which is essential for retaining the quality of the product.

**Percent Released After 24 h, \( \%R_{24} (Y3) \).** The estimates of the effects studied showed that the largest influence on the \textit{in vitro} release was due to the lipid/lecithin amount \((X3 = 3.91)\). The next largest effect was due to the amount of chitosan present in the formulation \((X4 = 1.82)\). Both effects were statistically significant at \( p < 0.001 \) and at \( p = 0.02 \) respectively. The lipid component of the lipid/lecithin mixture determines the fluidity of the membrane and the release of the entrapped drug depends on its permeability. Indeed, lipids such as fatty acid esters can help preserving membrane hydration by simply reducing the packing structure of the phospholipid layers, this in turn leading to better release of the drug from the core. Although chitosan was reported to reduce the membrane fluidity,\textsuperscript{36} its positive effect on the release could be due to the enhanced hydrophilicity of the vesicles enabling better contact with the dissolution medium. The estimates of the interaction \( X1 \times X4 \) and the drug load \( X1 \) were negative \((-2.078 \text{ and } -0.15 \text{ respectively})\). The response surface shown in Fig. 1c indicates that better release was obtained at high chitosan level but at lower drug load.

The results discussed above point to the likelihood of the nanoparticles structure consisting of a core entrapping the drug and a hydrophilic coat surrounding the lipid core.

The linear equation for \( \%R_{24} (Y3) \) can be written as follows:

\[
Y3 = 42.58 - 0.15 X1 + 3.91 X3 + 1.82 X4 - 2.8 X1 \times X4
\]

with an Intercept value of 42.58.

To further investigate the \textit{in vitro} release properties, the release profiles of all formulations were generated from the experimental data and are shown in Fig. 2. The dissolution profile of the free drug was also obtained and is added for comparison. More than 90% of the drug was released within the first 2 h from the drug solution. On the other hand, the release from the nanoparticles was lower and prolonged indicating that the nanocarrier was capable of controlling the release during the 24 h testing period. The release profiles of the drug from all prepared systems exhibited a biphasic shape with an initial immediate release taking place up to \(-2 \text{ h}\). This initial burst effect can be explained by the presence of PPL-HCL near the surface of the nanoparticles. The controlled release taking place over the remaining time period can be explained to have resulted from a diffusion process as well as from the decrease in the available drug as the time progressed. Biphasic release profiles have been reported for similar nanoparticles by Özcan et al., who suggested that drug molecules could be localized both in the core as well as in the surrounding layers.\textsuperscript{33}

**Mathematical Modeling of the Release Kinetics.** The \textit{in vitro} release data were fitted to zero order, first order, Higuchi equation and Korsmeyer–Peppas mathematical models. Regression analysis of \( Q(t) \) vs. \( t \) (zero order); log \( Q(t) \) vs. \( t \) (first order); \( Q(t) \) vs. square root of \( t \) (Higuchi); and log \( Q(t) \) vs. \( n \times \log t \) (Korsmeyer–Peppas), where \( Q(t) \) is the amount of drug released at time \( t \), was performed to obtain the correlation coefficients \( R^2 \). The results which were obtained showed poor fit to the first order model (\( R^2 \) values ranging

\[\text{Figure 2. In vitro release of PPL-HCL from nanoparticles and from drug solution.}\]
between 0.424 and 0.857) as well as to the zero-order model ($R^2$ values between 0.486 and 0.948). Fitting the data to the Higuchi equation gave better correlation with $R^2$ values ranging from 0.600 to 0.988. This suggests a release mechanism governed by diffusion. Fitting the data to the Korsmeyer-Peppas ($K/P$) model enables the calculation of the real value of the release exponent ($n$) rather than assuming a value of 1 (as in zero order) or of 0.5 (as in Higuchi model). The determination of the exact values of $n$ is useful when the release mechanism is not well defined and/or if more than one type of release mechanisms is involved. This is very often the case with nanoparticle carriers. The $n$ values obtained for all formulations were close to or smaller than 0.5 indicating that the drug release from the nanocarriers was overwhelmingly by Fickian diffusion without erosion of the nanoparticles. It should also be noted that $n$-values smaller than 0.5 are possible for polydisperse matrix systems such as nanoparticles.

Selection of Optimal PPL-HCL Nanoparticles Using Desirability Function. It is evident from the results obtained that the response properties were influenced by the variables in many ways. To overcome the difficulty of accounting of all the effects of the factors, the desirability function was applied. Moreover, by maximizing the desirability, an optimal formulation can be selected and recommended for further investigation including ex vivo and in vivo studies. The criteria for the selection were based on the constrains listed in Table 1.

The maximized desirability profile for the three responses identified the combination of the following levels of the five independent variables: 40; 2; 400; 0.2 and 5 for $X_1$; $X_2$; $X_3$; $X_4$ and $X_5$ respectively. This combination of the values of the factors corresponds to the system F28 with EE$\% = 53.62$, PS$ = 443.04$ nm and %R$_{24} = 56.11$, which are in close agreement with the experimentally obtained values (see Table 2). In addition to F28, F3 was also selected for further studies based on its optimum particle size.

Characterization of the Optimized Nanoparticles

Transmission Electron Microscopy (TEM). TEM images of PPL-HCLnanovesicles in Fig. 3 showed nearly spherical particles of both F28 (Fig. 3a) and F3 (Fig. 3b). By comparing the images, it is evident that in the case of F28 a layer of chitosan is visible surrounding the lipid/lecithin core. On the other hand, this contrasting layer appears incomplete and less visible in case of F3. This difference can be explained to be due to the lesser amount of chitosan present in F3 (100 mg) compared to F28 (200 mg) (Table 2). Other authors have shown similar evidence of the coating function of chitosan.$^{33}$

Differential Scanning Calorimetry (DSC). DSC thermograms of PPL-HCL, the individual components, F3 and F28 are illustrated in Fig. 4. PPL-HCL thermogram revealed a single endothermic peak at 165.6 $^\circ$C characterizing the melting point of the crystalline drug. The chitosan thermogram demonstrated an endothermic peak at 275 $^\circ$C. Equally, the DSC thermograms of GMS and Poloxamer 188 showed an endothermic peak at 62.4 $^\circ$C and 57.2 $^\circ$C, respectively, corresponding to their melting temperature. The absence of the PPL-HCL melting peak in the DSC thermograms of F3 and F28 indicated that the drug was present in an amorphous state within the nanoparticles.

Characterization of the PPL-HCL Loaded Nanoparticles Hydrogel pH Measurements. The pH values measured for F3 and F28 hydrogels were 5.22 ± 0.033 and 5.68 ± 0.016, respectively. These values were compatible with the skin pH which lies between 5.0-7.0.$^{37}$ Consequently both gels can be applied safely.

Rheological Measurements. Measurement of the rheological properties is necessary for topical formulation since it determines how formulation will spread and remain on the skin. Results showed that both formulations exhibited decrease in viscosity with shear rate, indicating non-Newtonian behavior as well as increase in shear stress with increasing shear rate indicative of pseudoplastic behavior. Thixotropic behavior of chitosan nanoparticles and the capability of their gels to enhance connection with the skin have been reported in the literature.$^{32}$

Cytotoxicity Studies

The effects of increasing concentrations of drug, F3 and F28 on HFB4 cells are shown in Fig. 5 (a-c). The results showed that the % toxicity of both systems dropped close to zero% at a concentration of 2000 $\mu$g/ml compared to a concentration of 500 $\mu$g/ml in the case of PPL-HCL. This indicated that the components of the nanoparticles can be characterized as biocompatible at the investigated concentration range.

Irritancy Study

In some cases, topical medication may cause irritation or some allergic reactions such as rash, swelling or scaling after their application on the skin. Regarding this issue, a precise consideration for
the effects of the constituents forming the topical formulation is a must to ensure safety and viability of the formulations. After visual scoring, it was observed that there was no apparent change on the exposed rat skin after 24, 48 & 72 h from the application of free PPL-HCL hydrogel, F3 and F28. All the rats showed no skin irritation potential or erythema (erythema score = 0).

In Vivo Study
Hemangeol solution, containing 4.28 mg/ml of PPL-HCl is approved to be used orally in the treatment of proliferating infantile hemangiomas. PPL-HCL being a beta blocker, thus, the systemic treatment requires vital signs monitoring (heart rate, blood pressure, sugar levels). The mechanisms of action of oral PPL-HCL in IH are not known, although it is hypothesized that they are a combination of vasoconstriction, inhibition of growth factors and increase in rate of hemangioma cell death (apoptosis).

Pediatricians however have administered PPL topically (off-label, since no market product exists) and have reported success in treating mild forms of IH.2 The rationale for a tailored topical PPL-HCL formulation is to deliver the drug topically to the skin through a carrier or reservoir suitable for drug deposition and accumulation near the walls of the hemangioma vessels, i.e., a topical treatment without metabolic changes (no first pass metabolism) or systemic side effects of the orally administered PPL-HCL.

PPL-HCL nanoparticles F3 and F28 were successfully designed with the goal to develop a candidate for the purpose discussed above. Combining lipid/lecithin and chitosan enabled bonding between the negatively charged core incorporating the drug and the positively charged chitosan forming a protective hydrophilic coating around the core.10 The lipid content provided fluidity of the

Fig. 4. DSC thermograms of (a) Chitosan, (b) GMS, (c) Poloxamer 188, (d) Lipoid S45, (e) PPL-HCL, (f) F3 and (g) F28.

Fig. 5. Effect of drug (a), F3 (b) and F28 (c) on HFB4 cells at different concentrations.

membrane and the chitosan imparted mucoadhesive properties needed to prolong residence time at the application site. Also, the particle sizes being in the nanorange provided large surface area which is needed to ensure sufficient contact between the nanoparticles and the skin enhancing deposition and accumulation of drug.

The capability of the optimized nanocarrier systems, developed in the present study, for depositing PPL-HCL in the skin with controlled passage through the skin was investigated using in vivo experiments.

**Skin Deposition Measurements.** In vivo studies using rats were conducted to determine the amount of drug deposited in the skin at different time intervals up to 24 h. The results, which are shown in Fig. 6a, are important because they provide information about the different behavior of the formulations in relation to PPL-HCL hydrogel on one hand, and between the two PPL-HCL nanoparticles loaded hydrogel on the other. The amount of deposited drug peaked at 2 h (F28) and 4 h (F3 and free drug). The maximum values were in the order F3max (74.40 ± 1.62 μg/cm²) > F28max (52.85 ± 0.74 μg/cm²) > Drug max (37.33 ± 0.98 μg/cm²). The differences were statistically significant between each formulation (F28, F3) and the PPL-HCL hydrogel (p < 0.05) as well as between the formulations (p < 0.05). It is also evident from Fig. 6a that the amount of deposited drug decreased with time. The amount of deposited drug remaining at the 24 h point ranged as follows: F2824h (36.63 μg/cm²) > F324h (26.16 μg/cm²) > Drug24h (15.70 μg/cm²). The differences were statistically significant (p < 0.05). The decrease can be attributed to some of the drug permeating through skin layers, because of skin saturation with time. The decrease is more pronounced in F3, whereas in F28 the amount of deposited drug remained almost constant following the decrease at around 4 h.

It is evident from Fig. 6a, that large amounts of the drug remained in the skin at the end of the testing period, indicating that the nanoparticles enhanced the deposition of the drug in the skin as a result of the greater hydrophobicity of the nanoparticles as well as its bioadhesive properties. To evaluate the differences in the behavior of the nanoparticles, the results illustrated in Fig. 7 were used to calculate the area under each skin deposition curve (AUC). The AUC values ranged in the following order: F28 (914.83 ± 1.94 μg h/cm²) > F3 (744.51 ± 0.01 μg h/cm²) > Drug (478.06 ± 0.42 μg h/cm²). The ANOVA analysis confirmed that the differences between F28 and drug hydrogel and between F3 and drug hydrogel as well as between F28 and F3 were all statistically significant (p < 0.05).

The AUC of F28 and F3 increased 1.91-fold and 1.56-fold respectively compared to the drug hydrogel. The enhanced deposition from the nanoparticles in relation to the drug hydrogel confirmed the capacity of lecithin/chitosan nanoparticles to promote accumulation of PPL-HCL in the skin. The large area of contact and the bioadhesiveness promoted their attachment to the skin and enabled the retention in the skin layers.

The higher retention capability of F28 compared to F3 (as illustrated by the larger AUC value of the former) can be explained by the higher amount of chitosan which renders the nanoparticles more hydrophilic. As such they could accumulate across skin layer which are more favorable to hydrophilic nanoparticles. This was supported by the results of the confocal laser scanning microscopy discussed below.

**Confocal Laser Scanning Microscopy (CLSM)**

CLSM is a helpful technique used for exploring and visualizing the allocation and penetration pattern of dissimilar pharmaceutical drug delivery systems across living tissues. CLSM images obtained after topical application of Rhb-labeled-F3 hydrogel, Rhb-labeled-F28 hydrogel and Rhb-labeled hydrogel (control) on the shaved backs of rats for 2 h are shown in Fig. 7. It can be visualized that the fluorescence was distributed differently relative to the control, which, as expected, retained low fluorescence intensity across the skin. The CLSM images of Rhb-labeled-F3 showed that more intense fluorescence was located near the surface of the skin than within the skin. On the other hand, the images of Rhb-labeled-F28 hydrogel showed that the fluorescence was more widely spread through the skin, indicating drug penetration via the intercellular route.

The fluorescence intensities for the Rhb-labeled-F3 hydrogel, Rhb-labeled-F28 hydrogel and Rhb-labeled hydrogel were calculated at increasing depths across the skin (i.e. at 0, 300, 600, 900 and 1200 μm) and are illustrated in Fig. 6b. The diagrams confirmed quantitatively the observations obtained from the images in Fig. 7 and showed that the difference in the penetration of the labeled nanoparticles across the skin was related to their composition.

The results of the CLSM study agreed with the results obtained from the skin deposition experiments (Fig. 6a). The nanoparticles consisting of lecithin/chitosan and a surface-active ingredient (Poloxamer 188) facilitated the skin deposition of PPL-HCL by enhancing the contact with the skin, hydrating the area of contact and interacting with the lipids in the skin. Our findings in the present study agree with the data obtained by Tan et al., who found
that because of the highly lipophilic nature of the lecithin, the drug carried cannot reach the inner more hydrophilic skin layers.\textsuperscript{10} Moreover, the formulation prepared in the form of nanoparticles tended to suppress transdermal delivery through the skin while enhancing topical delivery of the drug into the upper skin layers.\textsuperscript{38–40}

**Effect of Storage on the Properties of the Optimized Systems**

The results of the effect of storage at 4 °C over the 3-month period are given in Table 3 showing the changes in the entrapment efficiency and in particle size. It appears from the results in Table 3, that the EE\% values of both F3 hydrogel and F28 hydrogel remained unchanged during the 3-month period. The PS values of F28 remained stable within acceptable changes with a maximum increase of 3.85\% after 3 months of storage. The PS values of F3 indicated that the tendency for agglomeration increased after 2 and 3 months of storage, although the PS values remained within the nanorange. This can be explained by the fact that the higher amount of chitosan in the F28 formula compared to F3 resulted in more efficient coating of the lipid core which was also evident from the TEM microphotographs in Fig. 3.

**Conclusions**

Topical form of PPL for the treatment of IH is not available commercially and pediatricians have administered PPL topically off-label.

In this study, a viable topical delivery system for PPL-HCL was designed and optimized which enabled high accumulation of the drug in rat skin when compared to the drug solution.

However clinical trials involving IH patients are necessary to confirm its efficacy and safety when used topically. The PPL-loaded lecithin/chitosan nanogel of formula F28 developed in this study can be a promising candidate for clinical studies since it was tailored to target the skin in a controlled and reproducible manner. An approved topical form can benefit some IH patients by decreasing toxicity associated with the oral route.

**Disclosure Statement**

No conflict of interest was reported by the authors.

**References**
