Supplementary information

Design, Preparation and Evaluation of Liposomal Gel Formulations for Treatment of Acne: *In vitro* and *In vivo* Studies

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S1. Materials

Curcumin was procured from Chem-Impex International, Inc; USA, while lauric acid and azithromycin were obtained from Lobachemie,, India and Alembic Pharmaceuticals Limited, India respectively. Phospholipon® 90H, Phospholipon® 90G and 1, 2-Dioleoyloxy-3-trimethylammonium-propane chloride (DOTAP) were obtained as gift samples from Lipoid GmbH, Germany. Cholesterol, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were procured from Sigma Aldrich, USA. HeLa (human cervical cancer cell line), MDA-MB-231 (human breast adenocarcinoma cell line) and L929

(Mouse fibroblast cell line) were obtained from National Centre for Cell Science (NCCS), Pune, India. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Leibovitz's L-15 Medium, penicillin-streptomycin antibiotic solution and phosphate buffer saline (PBS) powder were obtained from Thermo Fisher Scientific, USA Tumor necrosis factor α (TNFα) and Interleukin 1β (IL1β) kits, chloroform, methanol and ultrapure water with resistivity 18 MΩ were obtained from Merck Millipore, Billerica, MA, USA Carbopol 980 was obtained from Lubrizol Corporation, USA Clindac A gel, clindamycin phosphate gel USP was obtained from Alkem Laboratories Limited, India. Disodium edetate was sourced from Kronox Life Sciences, India and ascorbic acid from Bajaj Health care, India. Propylene Glycol and triethanolamine were procured from Dow Chemicals, India and Panreac Applichem, Germany respectively. Supor 200 (25mm, 0.2µm) membrane was obtained from Pall Life Sciences, India. Brucella Agar, supplemental Brucella Agar, Hemin and vitamin K1 were obtained from Becton Dickinson and Company, USA Laked sheep blood was supplied by Hemostat Laboratories, CA, USA *Propionibacterium acnes* (*P. acnes* ATCC 6919) and *P. acnes* IHMA 775489 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and International Health Management Associates (IHMA, Inc, IL, USA) respectively.

S2. Morphological characterizations

High Resolution Transmission Electron Microscopy (HR-TEM) analysis

The morphological analysis of curcumin loaded liposomal suspension and gel containing liposomes was evaluated using high resolution transmission electron microscopy (HR-TEM, Technai G2, 200 kV, FEI, Italy). A drop of liposomes containing dispersion or gel was placed on a carbon coated grid and excess of the sample was removed with filter paper. Further, a drop of 2 % (w/v) uranyl acetate was applied over the grid for 1 min and excess of stain was removed. The grids were air dried, and samples were observed under the HR-TEM.

Field Emission Scanning Electron Microscopy (FESEM) and Atomic Force Microscope (AFM) analysis

The surface morphology of liposomes was evaluated by using field emission scanning electron microscope (FESEM, FEI Quanta 200F, Oxford-EDS system IE 250 X, U.S.A.). A drop of liposomal dispersion was taken on 10 mm round coverslip, freezed and further lyophilized. The freeze dried liposomes were observed under FESEM. The morphological characteristics of liposomes were also studied using atomic force microscope (AFM; Dimension icon Scan Asyst, Bruker, Germany). The sample for AFM was prepared similarly as FESEM and analyzed in tapping mode under multi-model nanoscope analyzer.

S3. Preparation of gel formulations

The sol to gel conversion was carried out by using Carbopol as a gelling agent. In brief, a varying amount of Carbopol 980 from 200-400 mg was added to the 20 mL of liposomal dispersion followed by addition of disodium edetate (20 mg) and ascorbic acid (10 mg). The mixture was stirred with a glass rod, and 200 mg of propylene glycol was added to provide emollient property to gel formulation. The basicity for gel preparation was adjusted by adding sufficient quantity of triethanolamine followed by stirring for 60 min. The gel formulations of lauric acid and azithromycin were prepared similarly as mentioned above.

S4. *In vitro* drug release

The cell, once placed in diffusion cell rack of the magnetic stirrer, having the receptor solution, was stirred magnetically at ~ 600 rpm. The temperature of the heating circulator bath maintained at 40 ºC to achieve membrane surface temperature of 32 ºC, which was measured before gel application using an infrared thermometer. The volume of receptor solution, isopropyl alcohol: water (40:60 v/v) was maintained up to the mark on the sampling arm. The

membrane, Supor 200 (25mm, 0.2µm) was equilibrated with receptor solution for at least 30 min before sample application. Curcumin loaded liposomal gel (300 mg) was evenly dispensed onto the membrane surface using a dosing syringe. Sequential aliquots of 300 µL of receptor solution were collected from each cell at different sampling time points of 0.5, 1, 2, 3, 4, 5 and 6 h, post sample application and replenished up to the mark of sampling arm with fresh stock of receptor solution. The aliquots were analyzed for curcumin estimation using HPLC method (**Table S4**). *In vitro* release rate was calculated using Higuchi equation by plotting the cumulative amount of drug release per unit area (µg/cm^2) against time (h$^{1/2}$) which yields a straight line, the slope of which gives the release rate.

S5. Efficacy of liposomal gel formulations in *P. acnes* induced acne using ear thickness rat model

Briefly, the inflammation in the ear of SD Rat was induced by intradermal injection *Propionibacterium acnes* ATCC 6919. *P acnes* induced inflammation at the site of injection mediated by the release of the pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β). The inflammation of pilosebaceous structure involving hair follicles and sebaceous glands led to the formation of comedones which was evaluated by histopathology study. On the day 0, 60 animals were weighed, and ear thickness of both the ears was measured using calibrated micrometer as the base line data. The inoculum was prepared by dilution of *P. acnes* culture in physiological saline having colony forming unit 5 × 10^8 per ml. All animals were anesthetized using isoflurane gaseous anaethesia before starting infection process. From this inoculums, 25µl (ca 1.25 × 10^7 per ear) were injected intradermally with 31 gauge needle into the mid ventral aspects of the right ear. The left ear of all rats was injected with 25µl of sterile 0.9% sodium chloride solution (saline). The injection of both ears was repeated for all animals. On day 1 ear thickness of all the animals was measured using a micrometer. Animals were assigned into 6 groups containing 8 animals each on the basis of ear

thickness. The selection criterion for animals was that the thickness of right ear be 1.4 ± 0.1 mm. The groups were divided as, Group A: normal control to demonstrate there was no contamination from other bacteria during experimental period, Group B: control (non-treated), Group C: blank liposomal gel (Placebo), Group D: curcumin liposomal gel, Group E: lauric acid liposomal gel, Group F: azithromycin liposomal gel, Group G: co-application of curcumin and lauric acid liposomal gels at the ratio 1:1. The ear thickness were repeatedly measured on days 3, 5, 7, 9, 11, 13, 15. The 100 mg of single active liposomal gel formulations of curcumin, lauric acid, and azithromycin were applied topically on the ventral side of the right ear of the animals of the respective groups from day 1 to 14 daily. In case of co-application of curcumin and lauric acid liposomal gels, 50 mg of each gel formulation was applied on right ear each day.

S6. Determination of encapsulation efficiency

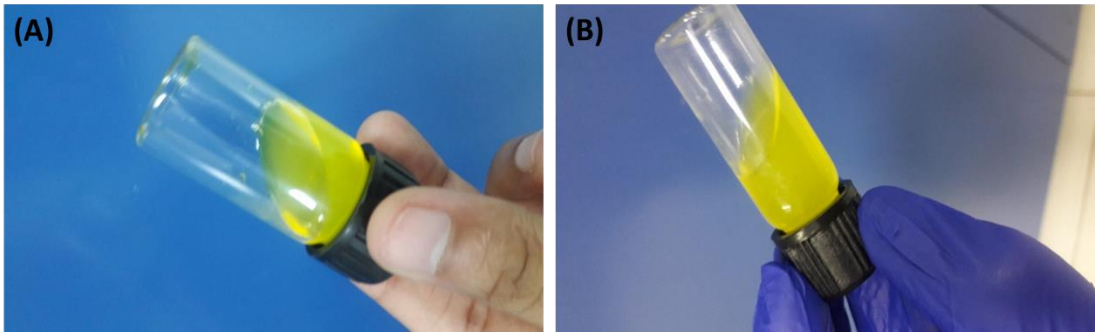
The entrapment efficiency of curcumin in liposomes was determined as per the previously published reports, where the unencapsulated curcumin was quantified, and entrapment of curcumin in liposomes was evaluated [1, 2]. Briefly, the curcumin loaded liposomal formulation was centrifuged at 14000 rpm, and the supernatant was decanted and further diluted with citrate buffer pH 5.0 and was examined immediately by using by UV-vis spectrophotometer (Shimadzu UV -1800, Japan). The amount of curcumin was calculated by comparing the absorbance (λ_{max} 427 nm) with the standard calibration curve plotted with pH 5.0 citrate buffer. The percent entrapment efficiency was determined by using following formula [3],

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \text{ X } 100$$

S7. Stability studies for curcumin liposomal formulation

Stability of curcumin loaded liposomes was assessed at 4 °C over a period of 60 days. The nanoformulation (10 mL) was flushed with nitrogen gas and kept in sealed vials for the above mentioned period. Samples were withdrawn after 30th and 60th day and analyzed for entrapment efficiency, particle size, PDI and zeta potential [4, 5].

Results:



Photographs for stability study assessment of curcumin loaded liposomal dispersion at 4 ^{o}C, (A) Initial sample (Day 1), Sample after 60 days.

S8. Cytocompatibility assay

Liposomes were evaluated for their cytocompatible nature for 24 h and 48 h in HeLa, MDA-MB-231, and L929 cells by using MTT cytotoxicity assay [6, 7]. In brief, 5 × 10^3 cells per well were seeded in a 96-well plate incubated in a CO_2 incubator at 37 °C. After reaching 70-80% confluency, blank liposomes at a concentration range of 40-1500 µg/mL were incubated with cells at 37 °C for 48 h. After 24 h and 48 h, media was removed, and fresh media of 200 µL was added in each well with further addition of 10 µL of MTT solution (5 mg/mL in PBS pH 7.4) and kept in a CO_2 incubator at 37 °C for 4 h. After 4 h, media with MTT dye was removed, and 200 µL of DMSO was added in each well to dissolve formazan crystals and kept at 37 °C for 15 min. Absorbance readings were measured at 540 nm on microplate spectrophotometer

(PowerWave XS2, BioTek Instruments, USA). The percent cell viability was calculated relative to negative control (PBS pH 7.4) and positive control (1% Triton X-100) using the following equation,

$$\text{Cell viability (\%)} = \frac{\text{Sample}_{540\text{nm}} - \text{Positive Control}_{540\text{nm}}}{\text{Negative Control}_{540\text{nm}} - \text{Positive Control}_{540\text{nm}}} \times 100$$

S9. Viscosity of gel

The viscosity of optimized gel formulation was measured at 25 ^{o}C with the help of viscometer (model DV-E, I-139 Brookfield Ametek, USA) by selecting suitable spindle number and rpm. In brief, 200 gm of gel preparation was kept in 250 ml beaker, and spindle groove of viscometer was dipped into the gel and rotated at different rpm. Viscosity was measured on the basis of Torque (%), spindle number, and rpm. (**Table S8**) [8, 9].

S10. Spreadability of gel

The spreadability of the optimized gel was investigated at room temperature using parallel plate technique with known weights [10-12]. In brief, 500 mg of sample was placed in the center of the acrylic plate, and furthermore, the sample was squeezed with round plate of known weight (10 g). Additional weights of 50 g and 100 g were included on round plate and kept for 5 min after which no more spreading was expected. Diameters of spread circles were measured in cm and were taken as comparative values for spreadability. The percent spread area was calculated as per the following equation [13] and plotted against additional weights.

$$\text{Spread area (\%)} = \frac{\text{Final area after spreading}}{2\ \text{cm}} \times 100$$

S11. Homogeneity of gel

The homogeneity of developed gel formulation was evaluated for any visible lumps and particulate matter. The grades were allotted to gel as +++ Good, ++ Fair, + Poor. Moreover, 200 mg of the gel was pressed between the index figure and the thumb to observe the consistency and homogeneous nature of the gel [14].

S12. pH of gel

A weighed amount of gel (2.0 g) was dissolved in 200 ml of milli-Q water, and pH of the gel was evaluated by using pH meter (CyberScan pH Tutor-I, UTECH, USA). The electrode was allowed to equilibrate with gel formulation for 2 min, and reading was noted [15]. The different batches of gel were checked in triplicates.

S13. Drug content uniformity

Drug content uniformity of curcumin loaded liposomal gel was evaluated for three independent batches as per the reported procedure [16]. In brief, 3.0 g of gel sample was taken from the top, middle and bottom layer of the container containing gel and further dissolved in 5 mL of methanol. The percent curcumin content w/w was determined from the standard calibration curve of curcumin at 427 nm using a UV-visible spectrophotometer.

S14. Polynomial equations obtained for tested responses from DOE

Entrapment efficiency (%) = $68.63 - 0.98 \times D + 13.89 \times FFT + 11.12 \times HydTe + 2.39 \times D^2 + 0.67 \times (D \times FFT) - 25.39 \times (FFT)^2 + 0.36 \times (D*HydTe) + 9.57 \times (FFT \times HydTe) - 17.55 \times (HydTe)^2$

Particle size (nm) = $95.74 + 71.95 \times D - 852.02 \times FFT - 329.80 \times HydTe - 13.24 \times D^2 + 23.46 \times (D \times FFT) + 986.02 \times (FFT)^2 - 120.26 \times (D*HydTe) - 234.94 \times (FFT \times HydTe) + 582.98 \times (HydTe)^2$

$$PDI = 0.32 + 0.008 \times D - 0.15 \times FFT - 0.09 \times HydTe - 0.04 \times D^2 - 0.004 \times (D \times FFT) + 0.28 \times (FFT)^2 - 0.05 \times (D*HydTe) - 0.07 \times (FFT \times HydTe) + 0.25 \times (HydTe)^2$$

$$\text{Zeta potential (mV)} = 8.80 + 3.53 \times D - 0.20 \times FFT - 0.04 \times HydTe + 1.69 \times D^2 + 0.02 \times (D \times FFT) - 0.42 \times (FFT)^2 - 0.10 \times (D*HydTe) - 0.47 \times (FFT \times HydTe) + 0.19 \times (HydTe)^2$$

Where, D - DOTAP (mg), FFT - Film formation temperature (°C), and HydTe - Hydration temperature (°C).

S15. Pharmacokinetics of curcumin loaded liposomal gel formulation

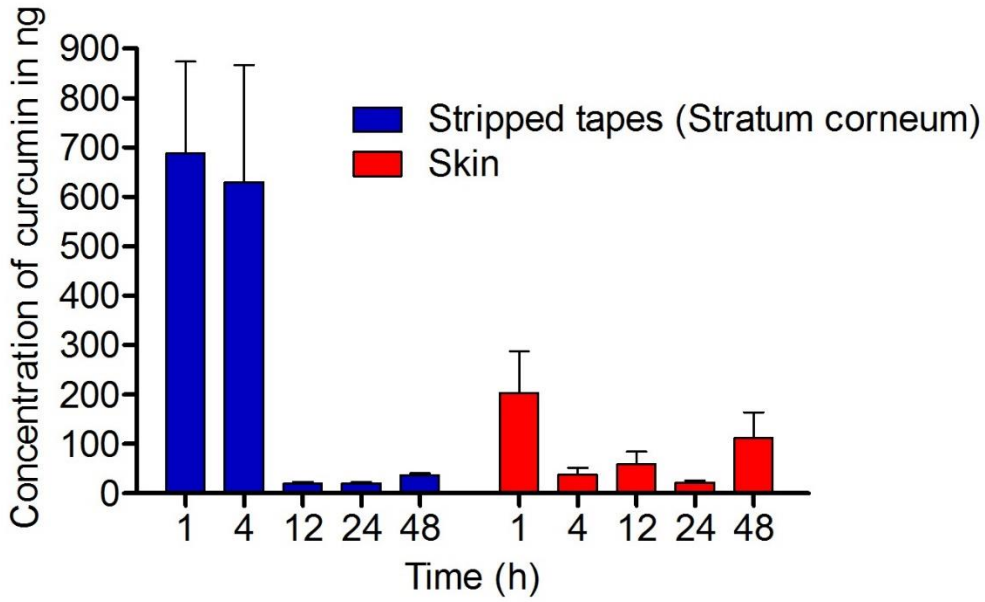
The pharmacokinetics for curcumin liposomal gel was conducted on male Sprague Dawley rats to investigate the distribution of curcumin (as a model drug) in stratum corneum, skin and plasma encountered by application of liposomal gel formulation. All the procedures for the study was followed as per the standard operating procedures followed at Preclinical Research and Development Organization, Pvt. Ltd (PRADO) and the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) as published in the Gazette of India, December 15, 1998. IAEC approved protocol number is IAEC – 18-050.

Hair on the dorsal flank region of each animal was removed by using electric clipper 1 day prior to topical applications. A gel of 100 mg (11.1 mg/cm^2) of topical gel was applied to the shaved flank area of an individual animal. After topical application of curcumin liposomal gel, blood (0.6 ml) was drawn from the retro-orbital plexus at 1, 4, 12, 24, and 48 h (n = 3 animals for each time interval) and collected in heparinized vials. Plasma was separated by centrifugation at 8500 rpm for 7 min at 4°C. Animals were euthanized, stratum corneum was removed by applying tape stripping to the animals at each time interval, followed by skin samples. The estimation of curcumin in plasma, stratum corneum and skin was determined by using Applied Biosystems LC-MS/MS system (Shimadzu Nexera, USA). Acetaminophen was used as an internal standard for bioanalytical method validation.

A summary of chromatographic and mass spectrometric conditions is as follows:

Column	Zorbax Eclipse XDB C18 50×2.1 mm, 5 µm
Mobile Phase	Acetonitrile: 0.2 % Formic acid in water, 40:60, v/v
Rinsing Solution	Acetonitrile: Water, 50:50, v/v
Flow rate	0.5 mL/min
Column oven Temperature	35 ºC ± 1.0 °C
Auto sampler Temperature	10 °C ± 1.0 °C
Injection volume	10 µL
RT Window	For Curcumin: 1.8 to 3.2 min
	For Acetaminophen: 0.15 to 0.40 min
Run time	5.0 minute

Results:



Pharmacokinetics of curcumin loaded liposomal gel. Where, curcumin was detected in ng/mL for stripped tapes (stratum corneum) and in ng/gm of skin for skin samples. (Mean ± SEM, n = 3).

Plasma samples from mice displayed negligible systemic absorption of curcumin. The detection of curcumin was found to be below the level of level of quantification. Such a type of observations were in good agreement with earlier published reports [17, 18]. T_{max} for curcumin was achieved within 1 h in both stratum corneum and skin post application of curcumin loaded liposomal gel, indicating quick penetration of nano-sized liposomes. Stripped

tapes (stratum corneum) showed C_{max} of 688.3 ng/mL and AUC_{0-t} of 5857.5 h×ng/mL, while skin samples exhibited C_{max} of 203.3 ng/gm and AUC_{0-t} of 2938.1 h×ng/gm.

S16. Evaluation of comedones and inflammatory parameters

On day 15, animals were sacrificed by CO_2 asphyxiation. Right ears from all tested rats were collected, and four out of eight ears were stored in 10 % buffered formalin solution. Small sections of ears were embedded in parafilm blocks and cut into 5 μm thin sections using fully automatic vibrating blade microtome (VT 1000 S, Leica, Germany). Sections were stained with Hematoxylin-Eosin dye and investigated under bright field illumination (Olympus IX73 microscope, USA). Hyperplastic follicles were scored as lesions (comedones) only if they lacked entirely of sebaceous remnants and contained a central eosinophilic plug of keratin. The remaining four ears were employed to evaluate the rat tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β). In brief, equal weight amount of ear tissues were homogenized in PBS pH 7.4 containing protease inhibitor cocktail. The tissue homogenates samples were centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was collected, and TNF-α and IL-1β were measured using ELISA kit. Ear tissue homogenate from uninfected rats was used as normal control.

Table S1. Plan of factors screening experiment on desired responses as per Placket-Burman design with center point repetition (n = 2).

Run No.	Pattern	Factors																			
		P 90G	P 90H	Ch	D	C	S	M:C	FFTe	FFP	FFS	FFTi	VDTi	VDTe	pH	B	Hyd S	HydTe	Hyd T	HPH Pr	HPH Cy
1	++++++++++++++++++++	180	25	15	15	6	30	0.75	60	250	75	15	3	30	6	30	90	70	3	1200	4
2	-++++-+-++--++--+-+-	140	25	15	15	6	10	0.75	30	250	75	5	1	30	6	10	60	70	1	1200	2
3	--++++-+-++--++--+-+	140	15	15	15	6	30	0.25	60	150	75	15	1	20	6	30	60	50	3	500	4
4	---++++-+-++--++--+-	140	15	5	15	6	30	0.75	30	250	45	15	3	20	4	30	90	50	1	1200	2
5	----++++-+-++--++--+	140	15	5	5	6	30	0.75	60	150	75	5	3	30	4	10	90	70	1	500	4
6	-----++++-+-++--++--	140	15	5	5	4	30	0.75	60	250	45	15	1	30	6	10	60	70	3	500	2
7	+-----++++-+-++--++-	180	15	5	5	4	10	0.75	60	250	75	5	3	20	6	30	60	50	3	1200	2
8	-+-----++++-+-++--++	140	25	5	5	4	10	0.25	60	250	75	15	1	30	4	30	90	50	1	1200	4
9	+-+-----++++-+-++--+	180	15	15	5	4	10	0.25	30	250	75	15	3	20	6	10	90	70	1	500	4
10	-+-+-----++++-+-++--	140	25	5	15	4	10	0.25	30	150	75	15	3	30	4	30	60	70	3	500	2
11	--+-+-----++++-+-++-	140	15	15	5	6	10	0.25	30	150	45	15	3	30	6	10	90	50	3	1200	2
12	+--+-+-----++++-+-++	180	15	5	15	4	30	0.25	30	150	45	5	3	30	6	30	60	70	1	1200	4
13	++--+-+-----++++-+-+	180	25	5	5	6	10	0.75	30	150	45	5	1	30	6	30	90	50	3	500	4
14	-++--+-+-----++++-+-	140	25	15	5	4	30	0.25	60	150	45	5	1	20	6	30	90	70	1	1200	2
15	--++--+-+-----++++-+	140	15	15	15	4	10	0.75	30	250	45	5	1	20	4	30	90	70	3	500	4
16	+--++--+-+-----++++-	180	15	5	15	6	10	0.25	60	150	75	5	1	20	4	10	90	70	3	1200	2
17	++--++--+-+-----++++	180	25	5	5	6	30	0.25	30	250	45	15	1	20	4	10	60	70	3	1200	4
18	-++--++--+-+-----+++	140	25	15	5	4	30	0.75	30	150	75	5	3	20	4	10	60	50	3	1200	4
19	+-++--++--+-+-----++	180	15	15	15	4	10	0.75	60	150	45	15	1	30	4	10	60	50	1	1200	4
20	-+-++--++--+-+-----+	140	25	5	15	6	10	0.25	30	250	45	5	3	20	6	10	60	50	1	500	4
21	+-+-++--++--+-+-----	180	15	15	5	6	30	0.25	30	250	75	5	1	30	4	30	60	50	1	500	2
22	++-+-++--++--+-+----	180	25	5	15	4	30	0.75	30	150	75	15	1	20	6	10	90	50	1	500	2
23	+++-+-++--++--+-+---	180	25	15	5	6	10	0.75	60	150	45	15	3	20	4	30	60	70	1	500	2
24	++++-+-++--++--+-+--	180	25	15	15	4	30	0.25	60	250	45	5	3	30	4	10	90	50	3	500	2
25	00000000000000000000	160	20	10	10	5	20	0.5	45	200	60	10	2	25	5	20	75	60	2	850	3
26	00000000000000000000	160	20	10	10	5	20	0.5	45	200	60	10	2	25	5	20	75	60	2	850	3

Table S2. Texture analysis parameters for curcumin loaded liposomal gel.

Sr. No.	Parameters	Settings
1	Test Mode	Compression
2	Pre-Test Speed	1.0 mm/s
3	Test Speed	0.5 mm/s
4	Post-Test Speed	10.0 mm/s
5	Target Mode	Distance
6	Force	100.0 g
7	Distance	20.0 mm
8	Strain	50.0 %
9	Trigger Type	Auto (Force)
10	Probe	P/25, 25 mm diameter
11	Points per second	200

Table S3. Texture analysis standard marketed clindamycin gel and curcumin loaded gel formulations with varying amount of Carbopol 980[a].

Texture analysis				
Gel with Carbopol amount	**Firmness (g), Peak positive force**	**Cohesiveness (g), Peak negative force**	**Consistency (g.s)**	**Index of Viscosity (g.s)**
Standard, Clindac A gel	39.83 ± 0.50	-26.46 ± 0.02	42.12 ± 0.68	-14.34 ± 0.02
Gel, 200 mg Carbopol	33.17 ± 2.11	-18.27 ± 0.39	35.26 ± 0.03	-10.63 ± 0.10
Gel, 250 mg Carbopol	42.63 ± 2.09	-25.26 ± 0.07	55.13 ± 0.63	-16.48 ± 0.17
Gel, 300 mg Carbopol	52.68 ± 3.15	-31.43 ± 0.56	62.76 ± 0.11	-19.43 ± 0.19
Gel, 350 mg Carbopol	59.29 ± 0.58	-30.84 ± 0.10	83.48 ± 0.11	-20.36 ± 0.11
Gel, 400 mg Carbopol	64.11 ± 2.06	-41.33 ± 0.08	90.78 ± 0.01	-23.31 ± 0.32
Liposomal dispersion	7.18 ± 0.61	-4.07 ± 0.06	11.51 ± 0.58	-0.33 ± 0.27

([a]Mean ± SD, n = 3)

Table S4. HPLC method for detection of curcumin from *in vitro* release.

Parameters	Selection
Analyte	Curcumin
Range	51.6 ng/mL to 10133.5 ng/mL
Column	Zorbax eclipse XDB C18 (50 × 2.1 mm, 5 µm)
Mobile Phase	0.1 % Formic acid in water: Acetonitrile; 60:40, *v/v*
Flow Rate	0.300 mL/min
Column Oven Temperature	45 °C ± 1.0 °C
Sample Cooler Temperature	10 °C ± 1.0 °C
Injection Volume	5 µL
UV Detection	425 nm
Retention Time	Curcumin: 3.0 to 4.0 min
Rinsing solution	Water: Methanol, 50:50 *v/v*
Total Run Time	5.0 min

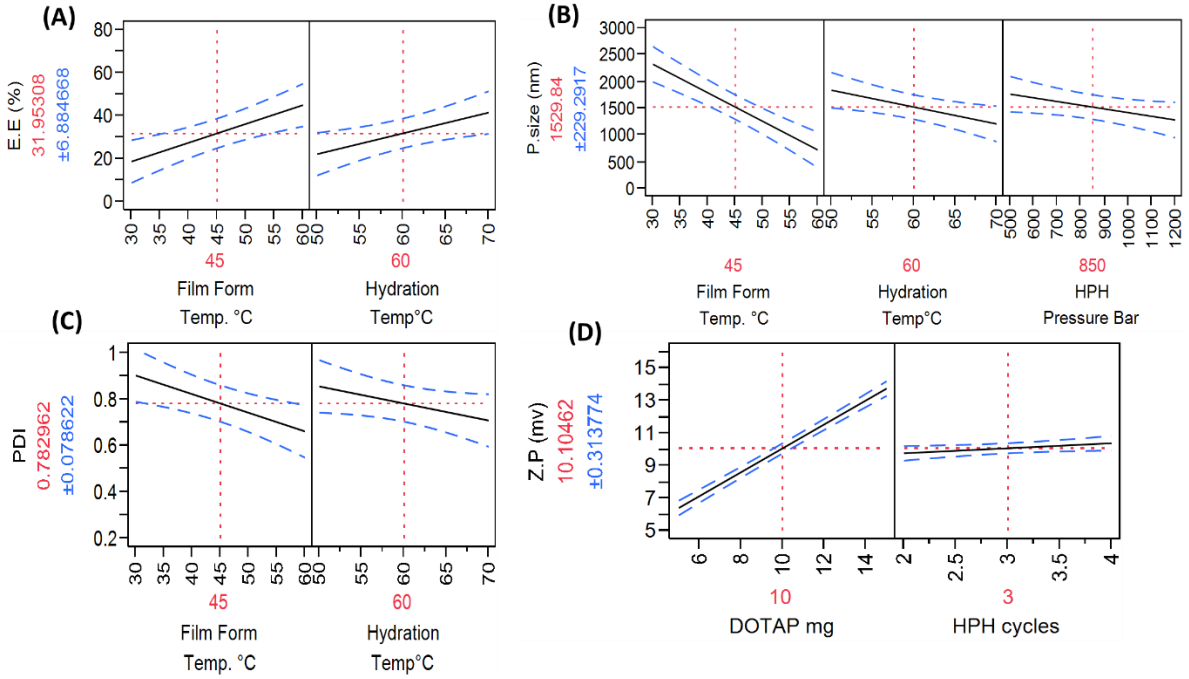


Figure S1. Prediction profilers obtained from PB design for liposomal formulation, (A) percent entrapment efficiency, (B) particle size (nm), (C) polydispersity index and, (D) zeta potential (mV).

Table S5. Augmented design response estimates.

Sr No	Terms[#]	Entrapment efficiency (%)		Particle size (nm)		Polydispersity Index		Zeta potential (mV)	
		Estimate	Prob>|t|	Estimate	Prob>|t|	Estimate	Prob>|t|	Estimate	Prob>|t|
1	D (5-15 mg)	-0.979	0.536	71.949	0.241	0.008	0.707	3.532	<0.0001*
2	FFT (30-60 °C)	13.888	<0.0001*	-852.019	<0.0001*	-0.146	<0.0001*	-0.195	0.107
3	Hyd T (50-70 °C)	11.123	<0.0001*	-329.796	<0.0001*	-0.093	0.0002*	-0.043	0.712
4	D*D	2.390	0.600	-13.235	0.939	-0.035	0.574	1.693	<0.0001*
5	D*FFT	0.673	0.682	23.459	0.709	-0.003	0.865	0.015	0.897
6	FFT*FFT	-25.394	<0.0001*	986.019	<0.0001*	0.282	0.0001*	-0.421	0.222
7	D* Hyd T	0.364	0.824	-120.257	0.065	-0.052	0.0287*	-0.104	0.397
8	FFT*Hyd T	9.573	<0.0001*	-234.934	0.0009*	-0.074	0.0030*	-0.475	0.0007*
9	Hyd T*Hyd T	-17.554	0.0007*	582.984	0.0025*	0.253	0.0005*	0.118	0.728

Where, Terms[#] represented as D: DOTAP, FFT: Film formation temperature and Hyd T: Hydration temperature. * indicates $p < 0.005$.

Table S6. ANOVA summary and model fitting statistics for entrapment efficiency (%), particle size (nm), polydispersity index and zeta potential (mV) from augmented design.

Entrapment efficiency (%)				
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	9	19110.67	2123.41	37.28
Error	24	1366.90	56.95	Prob > F
C. Total	33	20477.57		<0.0001*
Fitting summary				
R^2			0.93	
R^2 Adj.			0.91	
Root Mean Square Error			7.55	
Mean of Response			37.62	
Particle size (nm)				
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	9	34867645	3874183	46.34
Error	24	2006437	83602	Prob > F
C. Total	33	36874082		<0.0001*
Fitting summary				
R^2			0.945	
R^2 Adj.			0.925	
Root Mean Square Error			289.14	
Mean of Response			1285.44	
Polydispersity index				
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	9	2.19	0.24	22.13
Error	24	0.26	0.01	Prob > F
C. Total	33	2.45		<0.0001*
Fitting summary				
R^2			0.89	
R^2 Adj.			0.85	
Root Mean Square Error			0.10	
Mean of Response			0.71	
Zeta potential (mV)				
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	9	371.82	41.31	129.72
Error	24	7.64	0.32	Prob > F
C. Total	33	379.46		<0.0001*
Fitting summary				
R^2			0.98	
R^2 Adj.			0.97	
Root Mean Square Error			0.56	
Mean of Response			9.86	

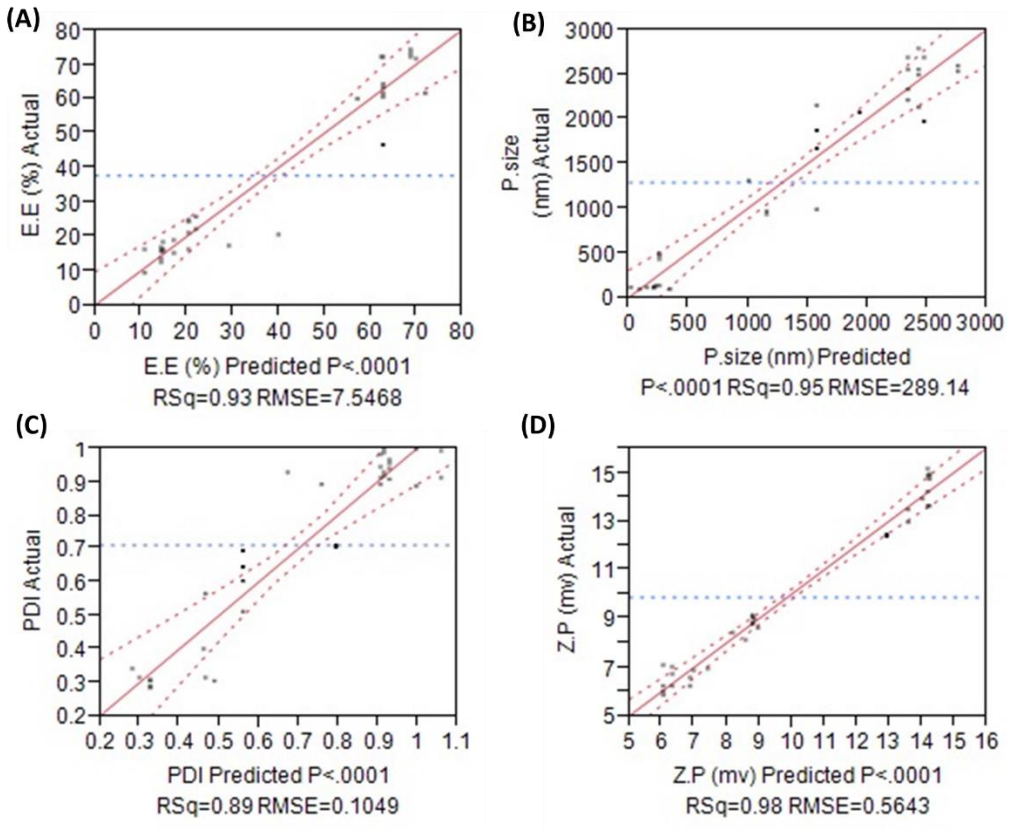


Figure S2. Correlation between measured and predicted responses from augmented design, (A) percent entrapment efficiency, (B) particle size (nm), (C) polydispersity index and, (D) zeta potential (mV).

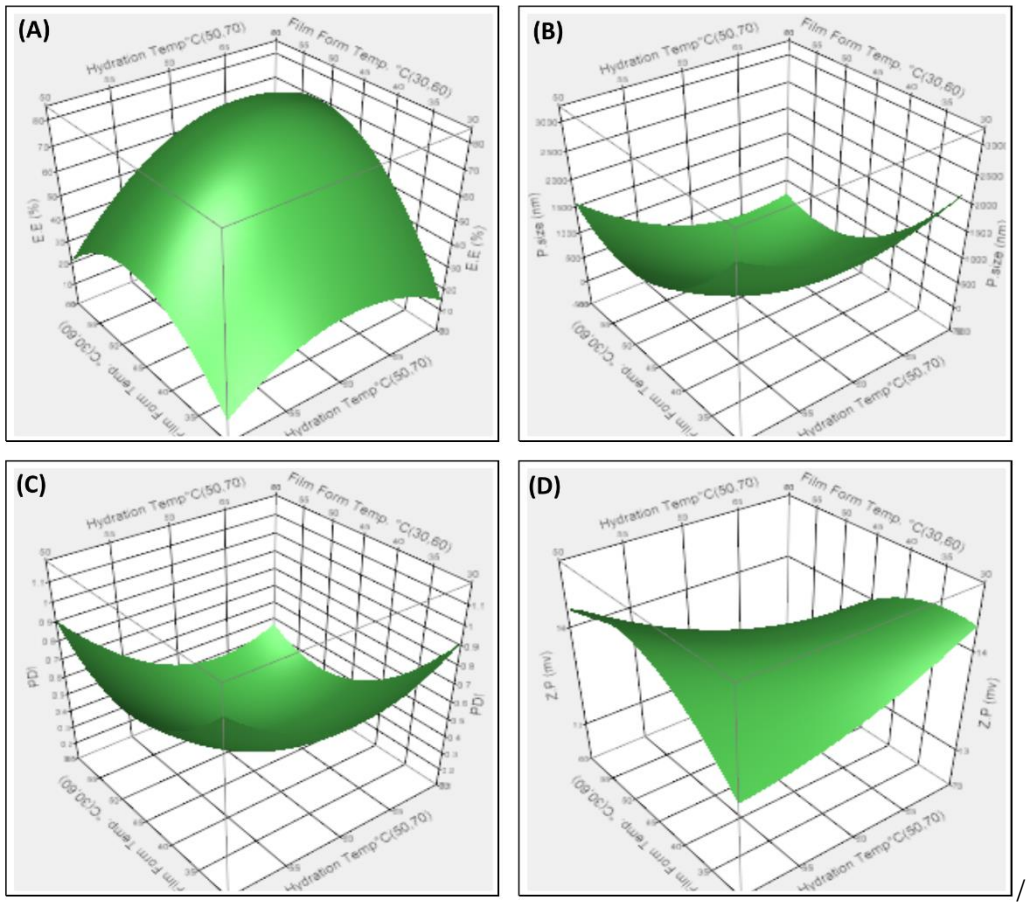


Figure S3. Surface response plots obtained from augmented design as quadratic effect, (A) entrapment efficiency (%), (B) particle size (nm), (C) polydispersity index and, (D) zeta potential (mV).

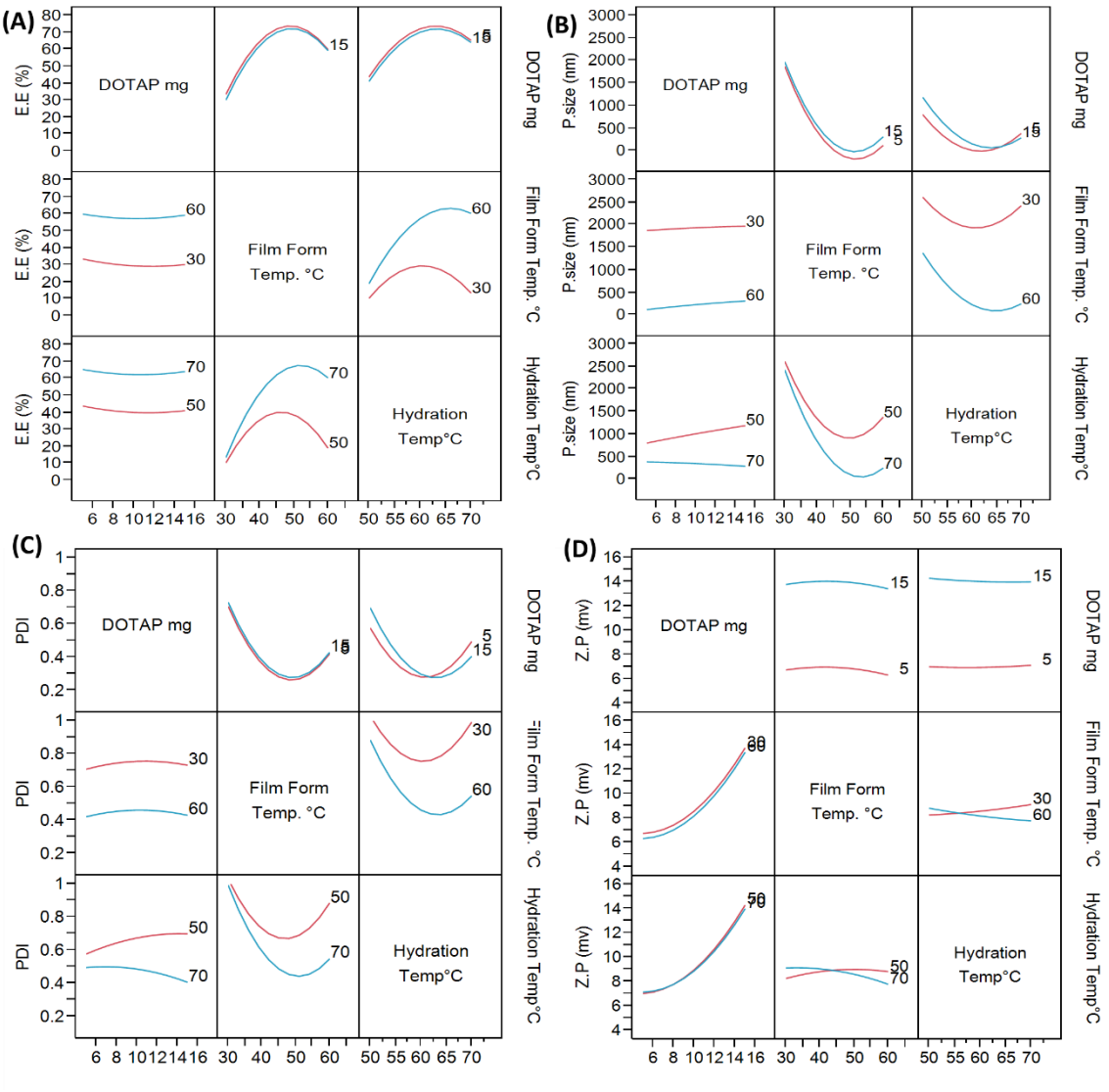


Figure S4. Interaction profilers for parameters in augmented design for, (A) entrapment efficiency (%), (B) particle size (nm), (C) polydispersity index and, (D) zeta potential (mV).

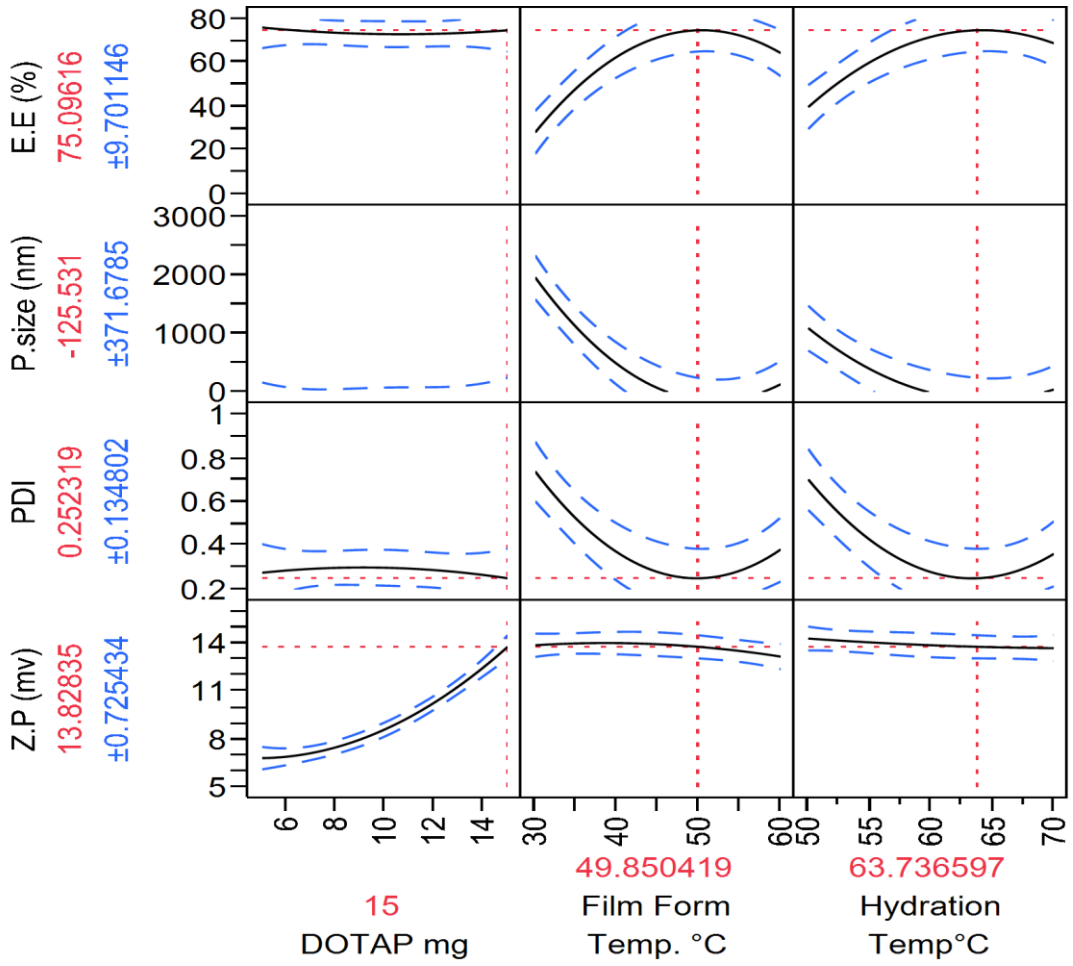


Figure S5. Prediction profilers based on target level of responses and maximum desirability criteria.

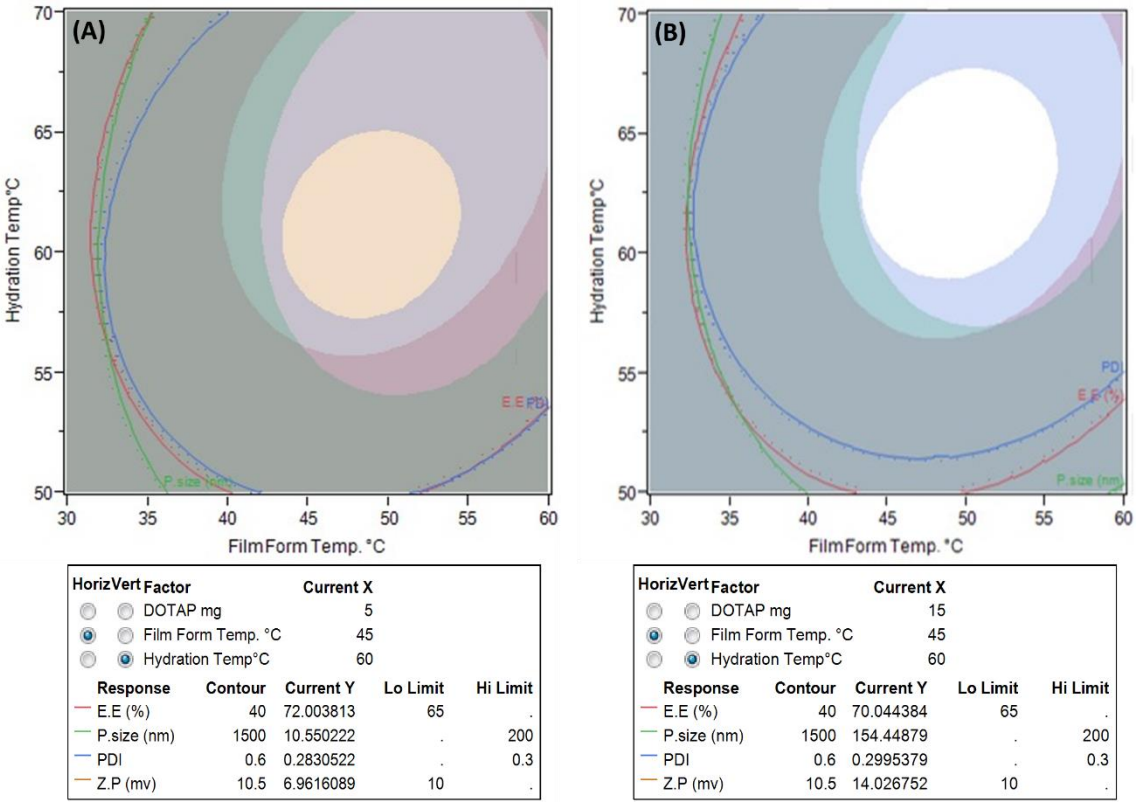


Figure S6. Contour plots with design space for target response using, (A) 5 mg DOTAP, (B) 15 mg DOTAP.

Table S7. Optimum parameters selected from DOE screening and design space for liposomal formulations.

Factors	P 90G	P 90H	Ch	D	C	S	M:C	FFT	FFP	FFS	FFTi	VDTi	VDTe	pH	B	Hyd S	Hyd T	Hyd Te	HPH Pr	HPH Cy
Selected values	160	20	10	15	5	20	0.5	50	200	60	10	2	25	5	20	75	64	2	850	3

Where, P 90G - Phospholipon 90G (mg), P 90H - Phospholipon 90H (mg), Ch - Cholesterol (mg), D - DOTAP (mg), C - Curcumin (%)/lauric acid (%)/azithromycin (%), S – Solvent, M:C - Methanol: Chloroform, FFT - Film formation temperature (°C), FFP - Film formation pressure (mBar), FFS - Film formation speed (rpm), FFTi - Film formation time (min), VDTi - Vaccum drying time (h), VDTe - Vaccum drying temperature (°C), pH - pH of buffer, B - Buffer volume (mL), Hyd S - Hydration speed (rpm), Hyd Te - Hydration temperature (°C), HPH Pr - HPH pressure (Bar), HPH Cy - HPH cycles (Cy).

Table S8. Viscosity determination of curcumin loaded gel formulation.

Spindle No.	rpm	Viscosity (centipoise)	Torque (%)
04	2.0	55200	55.3
	2.5	44960	56.2
	3.0	38000	57.0
	5.0	29300	58.5
	10.0	13260	66.3
05	2.0	57200	28.6
	5.0	25360	31.6

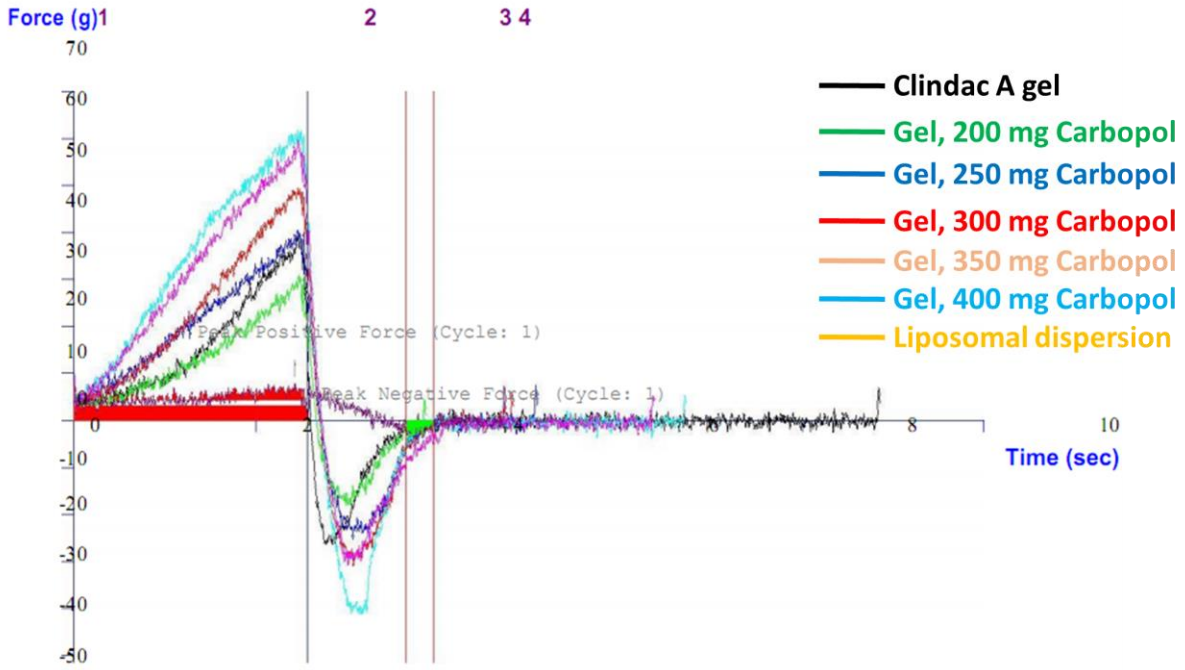


Figure S7. Texture analysis of commercial Clindac A gel, curcumin loaded liposomal gels with varying amount of Carbopol and curcumin loaded liposomal dispersion.

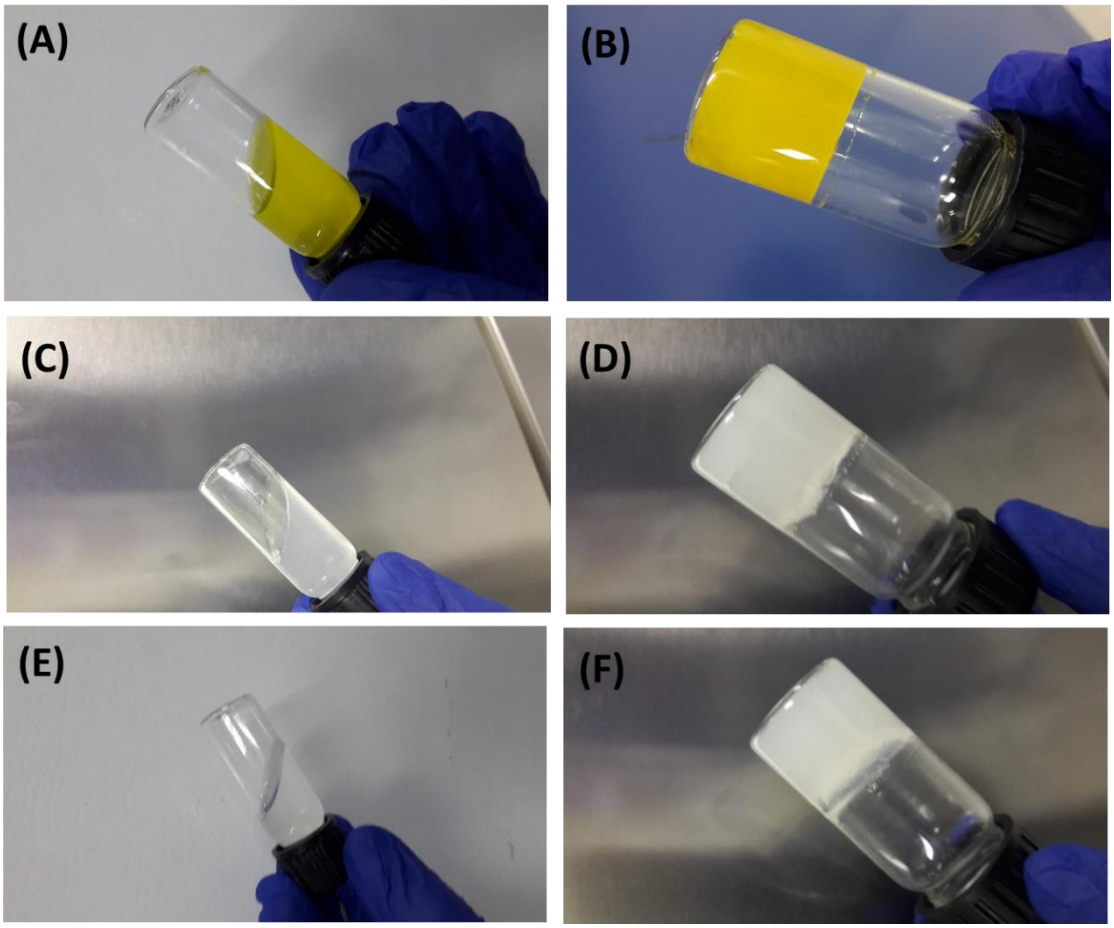


Figure S8. Conversion of sol to gel, (A and B) curcumin loaded liposomal dispersion and its gel respectively, (C and D) lauric acid liposomal dispersion and its gel respectively and (E and F) azithromycin loaded liposomal dispersion and its gel respectively.

Table S9. Content uniformity of curcumin loaded liposomal gel[a].

Sr. No.	Layer taken	Curcumin content (% w/w)
1	Top	107.61 ± 4.89
2	Middle	106.52 ± 6.67
3	Bottom	100.36 ± 3.94

([a]Mean ± SD, n = 3)

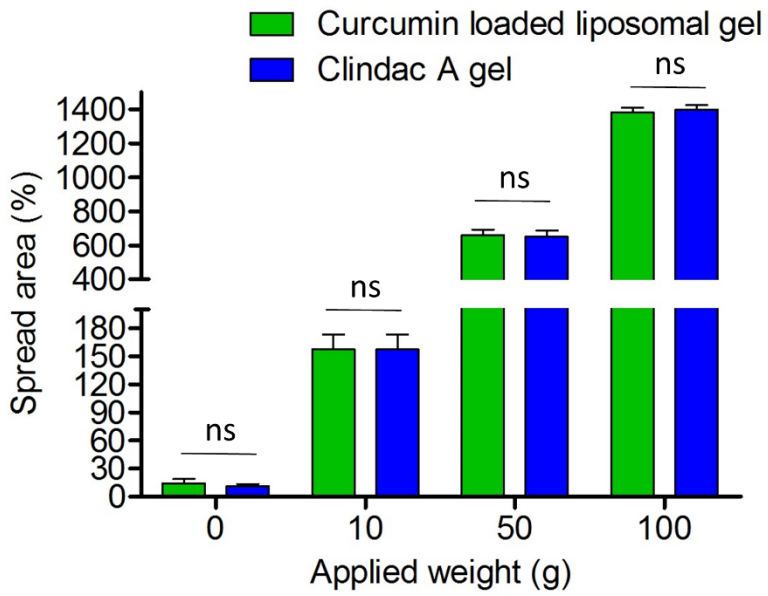


Figure S9. Percent spread area with increasing applied weight for curcumin loaded liposomal gel and commercial Clindac A gel. Mean ± SD, n = 3, where, “ns” indicate not significant.

Table S10. Cumulative amount of curcumin released versus square root of sampling time points for curcumin liposomal gel using Franz diffusion cell (FDC).

Sampling Time Points (h)	**Sampling Time Points ($h^{1/2}$)**	**Cumulative amount of analyte released (µg/cm^2)**					
		FDC-01	**FDC-02**	**FDC-03**	**FDC-04**	**FDC-05**	**FDC-06**
Predose	N/AP	0.0	0.0	0.0	0.0	0.0	0.0
0.5	0.707	1.2	0.9	0.9	1.1	1.1	1.2
1	1.000	4.6	3.2	3.1	3.2	3.9	4.2
2	1.414	10.1	6.7	6.4	6.3	7.9	8.9
3	1.732	13.1	9.4	9.1	8.8	10.3	12.2
4	2.000	15.3	11.5	10.7	10.9	11.8	14.5
5	2.236	16.8	13.1	12.0	13.0	13.3	16.2
6	2.449	18.0	14.2	13.1	14.2	14.3	17.4
	Release rate (µg/cm^2/$h^{1/2}$)	9.8115	7.8420	7.1603	7.6669	7.5705	9.5343
	R^2	0.9803	0.9967	0.9938	0.9992	0.9849	0.9906

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