Contents

5A.1. INTRODUCTION

With an objective to achieve therapeutic plasma levels of VPN via transdermal route, ultra-deformable liposomes (UDLs) were chosen for their capability to flow through the narrow intercellular pores and carry the active compound into deeper layers of the skin [1]. Out of several available methods for preparation, ethanol injection method was used for VPN. A systematic Quality-by-design (QbD) approach employing statistical design of experiments was utilized to exhaustively evaluate the impact of material attributes and process parameters on the critical formulation attributes [2].

5A.2. MATERIALS & METHODS

5A.2.1 Materials

Vinpocetine (VPN) was obtained as a gift sample from Covex S.A., Spain. Phospholipon 90G (Soya phosphatidylcholine, P90G) and Phospholipon 90H (hydrogenated Soya phosphatidylcholine, P90H) were kindly gifted by Lipoid GmBH, Germany. Sodium deoxycholate (SDC) was purchased from S.D. Fine Chemicals, Mumbai, India. Dialysis bags (MWCO, 12 kD) were purchased from HiMedia Labs Pvt. Ltd., Mumbai, India. Double distilled water was prepared in lab, filtered through 0.2μ membrane filter in glass bottle and consumed within a maximum of 7 days.

5A.2.2 Preparation of Vinpocetine loaded ultradeformable liposomes (VPN UDL)

VPN loaded UDL was prepared using ethanol injection method [3] with slight modification. Briefly, the VPN, P90G, P90H and SDC were dissolved in ethanol. Prefiltered distilled water was taken in a round bottom flask and continuously stirred on a magnetic stirrer at room temperature. Then organic phase was slowly added into aqueous phase using a 1ml syringe. The stirring was continued for next 15 minutes at room temperature and later, ethanol was evaporated using rotary evaporator. The UDL dispersion was centrifuged for 10 minutes at 5000 rpm and 15°C for the sedimentation of free drug. The supernatant UDL dispersion was carefully separated without disturbing the free drug pellet at the bottom. The separated UDL dispersion was stored in glass vials at room temperature till further analysis.

5A.2.2.1 Establishing Quality target product profile and Critical Quality Attributes

Based on the scientific, therapeutic, industrial and regulatory aspects, quality target product profile (QTPP) for VPN loaded UDL were established. Further, based on the prior knowledge, literature review and experiment trials, two response variables viz., vesicle size and drug entrapment were selected as critical quality attributes (CQA).

5A.2.2.2 Identification of Independent variables (factors) and qualitative risk assessment

Ishikawa diagram was used to demonstrate all the probable variables associated with development of VPN loaded UDL by ethanol injection method. These factors were qualitatively categorized as 'low, medium and high risk' based on their anticipated impact on CQA as described in Table 5A-1.

Table 5A-1. Quality risk assessment criteria

Low Risk	Factors with wide range of acceptability. No investigation required
Medium Risk	Acceptable risk. No adverse effect on product quality on small changes.
High Risk	Unacceptable risk. Acceptable range need to be investigated

Factors with low and medium risk were controlled by assigning constant levels based on literatures and preliminary trials.

5A.2.2.3 Quantitative risk assessment: Screening design

Factors with high risk were screened using 2-level fractional factorial design to statistically identify the critical factors and use them in main design to determine the control ranges (design space). Screening design was also utilized to assign constant levels of other non-critical factors. Minitab® 17.1.0 was used to generate a randomized design matrix based on which experimental batches were prepared and evaluated for CQA. Software based Pareto charts were utilized to determine critical factors while the main effect charts were utilized to decide the optimum levels of non-critical factors. Methods used for estimation of CQA are as follows

5A.2.2.3.1 Vesicle size and size distribution

UDL dispersions were diluted ten times with pre-filtered distilled water, transferred to disposable sizing cuvette and analyzed by dynamic light scattering (DLS) using Nano-ZS Zetasizer, Malvern Instruments Ltd., UK for vesicle size (VS) and poly-dispersity index (PDI). The instrument analyzes angular scattering of a laser beam during its passage through the dispersed UDL sample and use the Mie theory of light scattering to calculate the mean diameter of liposomes.

5A.2.2.3.2 Drug entrapment

Samples from UDL dispersions (0.1 ml) were dissolved and suitably diluted in acetonitrile-methanol (volume ratio, 2:8) and analyzed using uv-visible spectrophotometric method described earlier in chapter 3. Drug entrapment (%) was then calculated using **Eq. 5A-1**.

$$\text{Drug entrapment}\,(\%) = \frac{\text{Entrapped drug (mg)}}{\text{Total drug taken (mg)}} \times 100 \qquad \textbf{Eq. 5A-1}$$

5A.2.2.4 Formulation optimization by combined D-optimal response surface design

Combined D-optimal response surface design was applied to exhaustively investigate the relationship between critical factors and CQA with less number of experimental batches while handling mixture components and other numeric factors simultaneously [4]. Design Expert® 7.0.0 was used for generating the randomized design matrix and statistical evaluation of experimental data to achieve optimization solution and creating the design space. Suitability of model suggested by the software and identification of significant model terms were decided based on analysis of variance followed by F-test. Insignificant model terms were later removed to simplify the mathematical equations for calculation of CQA. The relationship between critical factors and CQA was explored using contour and 3-D response surface plots. Desirability criteria was defined based on QTPP and design space was created to obtain final optimized batch. Three batches were prepared with optimized composition for model verification.

5A.2.3 *In vitro* characterization of optimized VPN UDL

5A.2.3.1 Shape and surface morphology

The VPN UDL (VPN loaded UDL with optimized composition) were evaluated for shape and surface characteristics using transmission electron microscopy. Dispersion was spread on a carbon-coated grid, excess solution was removed and the grid was dried under infrared lamp. It was negatively stained with 2% phosphotungstic acid (PTA) and again dried under Infrared lamp. Transmission electron microscope (CM 200,

Philips, Netherlands) with operating voltage range of 20-200 kV was used to visualize liposomes at suitable enlargement with an accelerating voltage of 20 kV.

5A.2.3.2 Zeta potential

VPN UDL dispersion was diluted ten times with pre-filtered distilled water, transferred to disposable folded capillary cells and analyzed for zeta potential using Nano-ZS zetasizer. The instrument utilizes Smoluchowski equation that calculates zeta potential based on amount of doppler shift occurring due to electrophoretic mobility of colloidal particles in response to the electric field applied to the dispersion.

5A.2.3.3 In vitro drug release study

The *in-vitro* drug release from optimized VPN UDL was evaluated using a Franz-type diffusion cell with an effective surface area of 3.14 cm^2 and a receptor chamber volume of 15 ml. Pre-activated dialysis membrane (MWCO, 12 kD) was mounted, as a permeation barrier, between donor and receptor chambers of diffusion cell. The receptor chamber was filled with a mixture of ethanol and double distilled water (ratio 3:7) as a diffusion media and allowed to equilibrate for half an hour. The optimized batches containing 1 mg of drug were transferred to donor chambers of diffusion cells. The diffusion medium was continuously stirred using a magnetic stirrer. 1 mL sample was withdrawn from sampling arm of diffusion cell at each time point up to 24 hours and equal volume of fresh diffusion media was added to maintain total receptor volume. Quantitative estimation of drug was performed by HPLC method at 280 nm detection wavelength as described earlier in chapter 3. The kinetics of drug release was then evaluated by fitting the data in various mathematical models and comparing their regression coefficient (R^2) values [5].

5A.3. RESULTS & DISCUSSION

5A.3.1 Preparation and optimization of VPN loaded UDL

5A.3.1.1 Establishing QTPP and CQA

Various QTPP elements and their targets were defined and presented with justification in Table 5A-2. Vesicle size and drug entrapment were identified as critical in governing the product quality and need to be within known limits to attain the pre-defined QTPP. Thus, these two characteristics were selected as CQA.

Table 5A-2. QTPP elements with justification for VPN loaded UDL

QTPP element		Target	Justification
Route of administration		Transdermal	Avoid first pass metabolism and achieve prolonged action
Dosage form		Ultradeformable liposome	Better skin permeability and controlled drug release
Formulation quality attributes	Vesicle size[#]	Minimize (~100 nm)	To ensure better permeation and drug release
	Polydispersity Index	Minimize (< 0.3)	To ensure uniformity of size and related characteristics.
	Zeta potential	> ±30 mV	To ensure stability of the dispersion
	Surface characteristics	Spherical, smooth	To ensure better permeation
	Drug entrapment[#]	Maximize	To minimize drug wastage for cost-effectiveness
	Shape	Ultradeformable	For better skin permeation
	In vitro Drug release behavior	Prolonged for 24 hours	To ensure controlled drug release for desired duration
Ex vivo permeability		Better transdermal flux	To ensure PK/ PD comparable to marketed formulations
Stability		NLT 1 months	To ensure stability till incorporation in final dosage form
Safety		Non-toxic & Non-irritant to skin	To ensure safety of the final formulation
Pharmacokinetics		Similar or better than oral suspension	For bioequivalence requirement
Pharmacodynamics			To demonstrate therapeutic efficacy

[#] Critical quality attributes

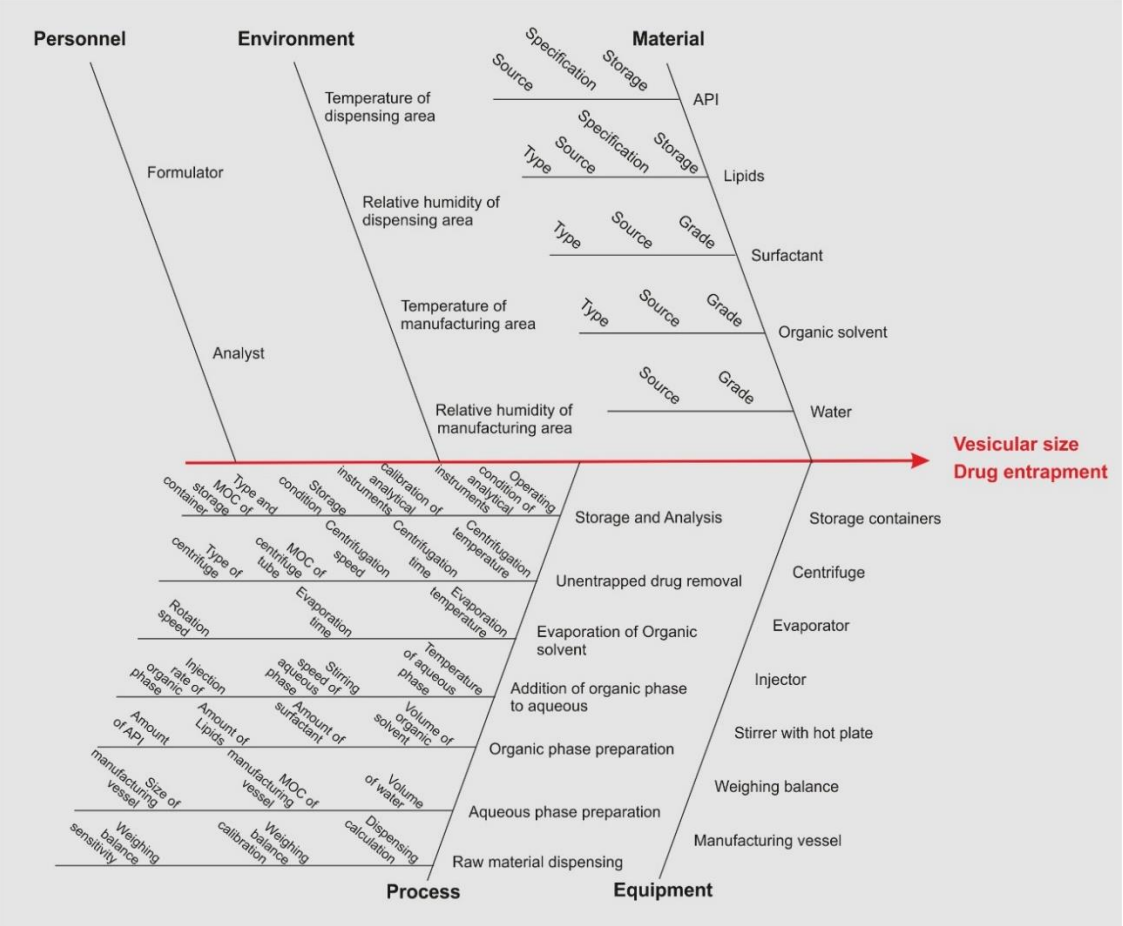

Fig. 5A-1. Ishikawa diagram showing probable variables that may influence the CQA

5A.3.1.2 Identification and qualitative assessment of Independent variables (factors)

All the probable variables associated with development of VPN loaded UDL by ethanol injection method were identified during the brainstorming sessions and categorized in to Material, Process, Equipment, Personnel and Environment. An ishikawa diagram illustrating the cause and effect relationship among identified variables and CQA was constructed (**Fig. 5A-1**).

5A.3.1.3 Qualitative risk assessment

The risk associated with all the identified factors were evaluated based on the predefined criteria (**Table 5A-1**) and the result is presented in **Table 5A-3**. Factors with low and medium risk were assigned with the best available constant levels based on literature and preliminary trials to ensure no or negligible impact of these factors on CQA. These constant levels are also listed in **Table 5A-3**.

Table 5A-3. Qualitative risk assessment of independent variables

Factors	Process step	Impact on CQA	Constant levels
Source and specifications of API	Raw material Selection and Storage	Low risk	Authentic source with COA
Storage condition of API		Low risk	Stored at recommended condition
Type of Lipid with low Tg		Low risk	Phospholipon 90G
Type of Lipid with high Tg		Low risk	Phospholipon 90H
Source and specifications of lipids		Low risk	Authentic source with COA
Storage condition of Lipids		Low risk	Stored at recommended condition
Type of Surfactant		Medium risk	Sodium deoxycholate
Source and specifications of Surfactant		Low risk	Authentic source
Storage condition of Surfactant		Low risk	Stored at recommended condition
Type of Organic solvent		Medium risk	Ethanol
Source and specifications of Organic solvent		Low risk	Authentic source
Source of water		Low risk	In house
Grade of water		Low risk	Filtered (0.2µ) Double distilled
Weighing balance sensitivity	Dispensing	Low risk	0.1 mg
Weighing balance calibration		Low risk	Calibrated
Temperature and RH of Dispensing Area		Low risk	25±3°C, Ambient RH
Dispensing calculations		Low risk	Calculated using excel and verified
Type, Size and Material of Construction (MOC)	Manufacturing Vessel	Low risk	50 mL round bottom flask of class A borosilicate glass
Temperature and Relative humidity	Manufacturing Area	Low risk	25±3°C, Ambient RH
Volume of Water	Aqueous phase preparation	Low risk	5 mL
Amount of API	Organic phase preparation	High risk	To be optimized
Amount of Lipids		High risk	To be optimized
Amount of Surfactant		High risk	To be optimized
Volume of Organic solvent		High risk	To be optimized
Calibration of Injector and stirring equipment	Addition of Organic phase to aqueous	Low risk	Calibrated
Injection Rate of Organic phase		High risk	To be optimized

Factors	Process step	Impact on CQA	Constant levels
Stirring speed of Aqueous phase		High risk	To be optimized
Temperature of Aqueous phase		Low risk	60 °C
Evaporation time	Evaporation of Organic solvent	Low risk	1 hours
Evaporation temperature		Low risk	60 °C
Stirring speed during evaporation		Low risk	100 rpm
Type of Centrifuge	Unentrapped drug removal	Low risk	Cooling centrifuge
Type and MOC of Centrifuge tube		Low risk	15 mL conical-bottom glass tube with screw cap
Centrifugation speed		Medium risk	5000 rpm
Centrifugation time		Low risk	10 minutes
Centrifugation temperature		Low risk	15°C
Type and MOC of Storage container	Storage and Analysis	Low risk	20 mL flat-bottom glass vial with screw cap
Storage condition		Medium risk	Room Temperature
Calibration of Analytical Instruments		Low risk	Calibrated
Methods used of Analysis		Low risk	Validated
Formulator	Personnel	Low risk	Common for all experiments and analysis
Analyst		Low risk	

Factors with high risk were carried forward for quantitative risk assessment.

5A.3.1.4 Quantitative risk assessment: Screening Design

Factors with high risk were statistically assessed by 2-level fractional factorial screening design. The low (-1) and high (+1) levels of all the independent variables were decided based on literatures as well as preliminary trials and are listed in **Table 5A-4**. The randomized design matrix of 17 experimental batches (including one center point) was generated using Minitab® 17.1.0 statistical software and presented in **Table 5A-5**. The data were statistically processed by Minitab software to generate pareto, normal and main effect plots for both CQA considering $P < 0.05$ as a level of significance.

Table 5A-4. Various material attributes and process parameters along with their levels for screening by fractional factorial design

Independent variables		Unit	Levels	
			-1	+1
A:	Amount of Lipid-1 (Phospholipon 90G)	mM	10	15
B:	Amount of Lipid-2 (Phospholipon 90H)	mM	5	10
C:	Amount of Drug	Mole %	5	10
D:	Surfactant concentration	Mole %	5	15
E:	Rate of organic phase addition	mL/min	0.5	1.0
F:	Stirring speed	rpm	500	1000
G:	Volume of organic solvent	mL	1	2

Table 5A-5. Randomized batch matrix and resulting CQA for screening design

Batch no.	Run order	Independent Variables							CQA	
		A	B	C	D	E	F	G	Vesicle size (nm)	Drug Entrapment (%)
S_1	02	-1	-1	-1	-1	-1	-1	-1	95.39	85.80
S_2	03	+1	-1	-1	-1	+1	-1	+1	84.12	91.90
S_3	10	-1	+1	-1	-1	+1	+1	-1	102.76	68.35
S_4	16	+1	+1	-1	-1	-1	+1	+1	96.57	84.67
S_5	11	-1	-1	+1	-1	+1	+1	+1	105.64	76.22
S_6	13	+1	-1	+1	-1	-1	+1	-1	88.81	83.79
S_7	15	-1	+1	+1	-1	-1	-1	+1	106.92	63.95
S_8	14	+1	+1	+1	-1	+1	-1	-1	98.36	76.14
S_9	09	-1	-1	-1	+1	-1	+1	+1	87.65	84.98
S_{10}	06	+1	-1	-1	+1	+1	+1	-1	82.52	92.12
S_{11}	08	-1	+1	-1	+1	+1	-1	+1	99.58	68.48
S_{12}	04	+1	+1	-1	+1	-1	-1	-1	89.24	80.09
S_{13}	01	-1	-1	+1	+1	+1	-1	-1	93.48	76.58
S_{14}	07	+1	-1	+1	+1	-1	-1	+1	87.89	80.99
S_{15}	12	-1	+1	+1	+1	-1	+1	-1	102.63	62.04
S_{16}	17	+1	+1	+1	+1	+1	+1	+1	91.03	69.55
S_{17}	05	0	0	0	0	0	0	0	93.84	76.54

Pareto and normal charts (**Fig. 5A-2**) clearly showed that amount of lipids, drug and surfactant had a significant effect on vesicle size of resulting liposomes. Similarly, amount of lipids and drug showed significant impact on drug entrapment. Owing to these observations, amount of lipids, drug and surfactant were selected as CMA for the final optimization step.

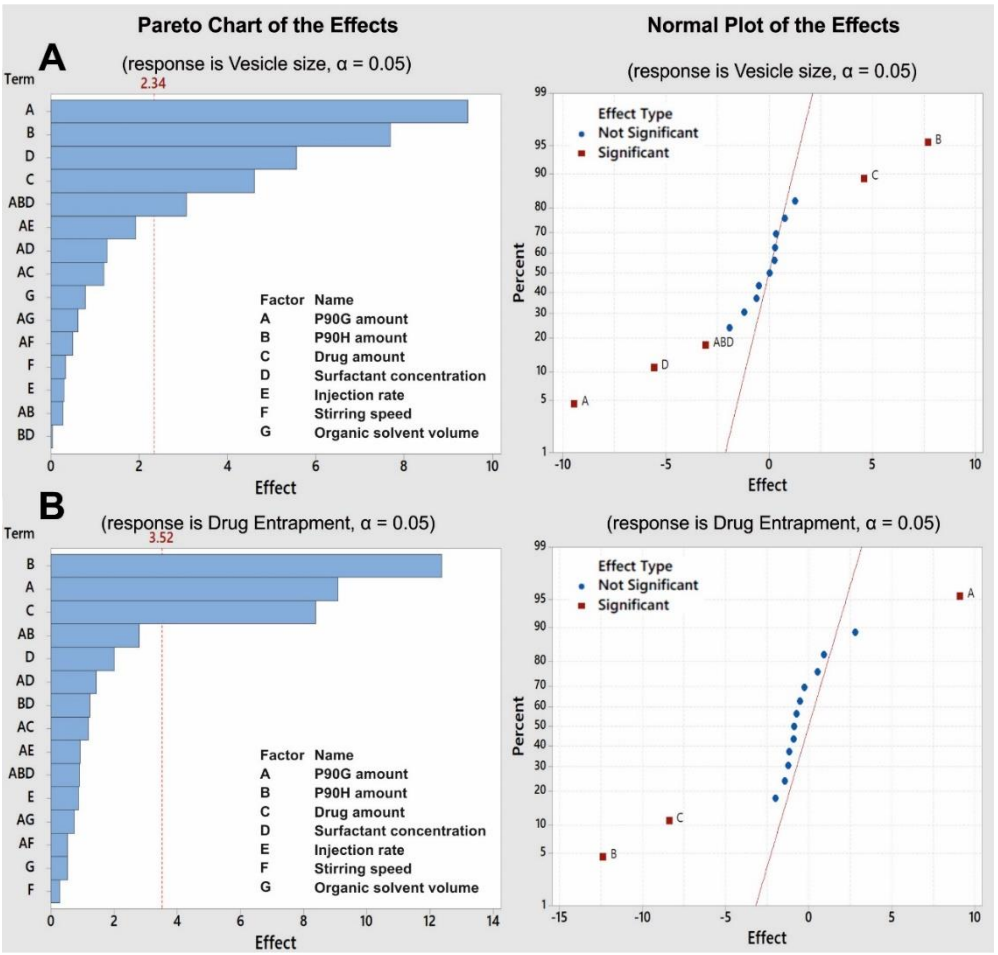

Fig. 5A-2. Pareto and Normal plots for A. Vesicle size and B. Drug entrapment

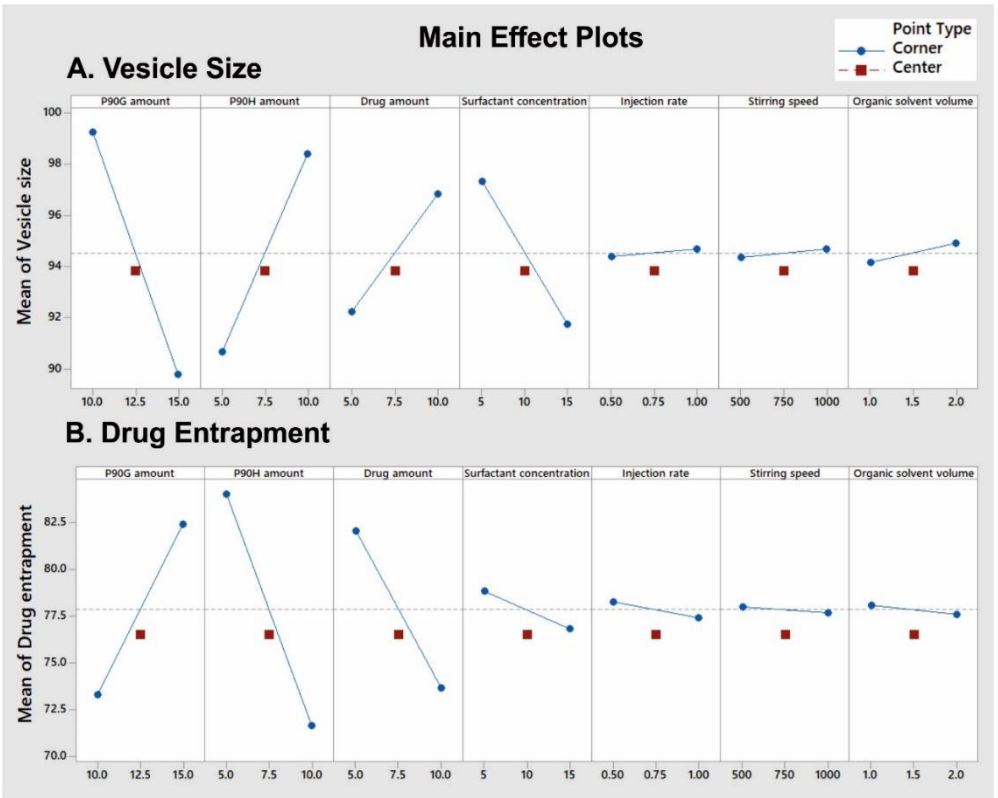

Fig. 5A-3. Main effect plots for A. Vesicle size and B. Drug entrapment

The influence of injection rate, stirring speed and organic solvent volume was found insignificant on both CQA. Hence, main effect plots were utilized to decide the constant level of these factors for final optimization step. Considering the positive impact on vesicle size and drug entrapment (**Fig. 5A-3**), lower levels of all three factors (injection rate, 0.5 mL/min; stirring speed, 500 rpm and organic solvent volume, 1 mL) were chosen.

5A.3.1.5 Formulation optimization by combined D-optimal response surface design

Based on the results of screening design, four CMA were identified and their relationship with CQA were exhaustively investigated using combined D-optimal response surface design. Combined D-optimal design was selected as it allowed the evaluation of lipid mixture together with other numerical variables viz., drug amount and surfactant concentration. The low (-1), medium (0) and high (+1) levels of all four CMA are listed in **Table 5A-6**. Amount of P90G and P90H in lipid mixture was varied in such a way to keep the total lipid level at 20 mM.

Table 5A-6. Various critical material attributes along with their levels for screening by Combined D-optimal design

Independent variables (CMA)		Unit	Levels		
			-1	0	+1
A:	Amount of P90G	mM	10	15	20
B:	Amount of P90H	mM	0	5	10
C:	Surfactant concentration	Mole %	5	10	15
D:	Amount of Drug	Mole %	5	7.5	10

A randomized matrix of twenty eight batches was generated by Design-Expert and presented in **Table 5A-7**. These batches were formulated as per their run order and evaluated for CQA using the methods described earlier. **Table 5A-7** also represents the resulting CQA of these batches. Based on the experimental data of vesicle size, software suggested quadratic model for mix order and linear model for process order. Analysis of variance (ANOVA) was performed by the software for suggested models. Model terms with a p-value less than or equal to 0.05 (α-level) were considered as significant while terms with higher p-value

were considered insignificant. Hierarchy based removal of insignificant model terms was done to simplify the model.

Table 5A-7. Randomized design matrix for Combined D-optimal response surface design

Batch no.	Run order	Independent Variables				CQA	
		A	B	C	D	Vesicle size (nm)	Drug entrapment (%)
F_1	18	10.0	10.0	15.0	5.0	102.24	75.93
F_2	14	20.0	0.0	15.0	5.0	83.62	72.74
F_3	5	20.0	0.0	5.0	10.0	94.77	63.22
F_4	28	10.0	10.0	5.0	10.0	109.57	60.84
F_5	13	10.0	10.0	5.0	6.5	102.29	67.78
F_6	3	20.0	0.0	5.0	5.0	89.46	64.24
F_7	9	20.0	0.0	15.0	10.0	86.80	69.36
F_8	8	20.0	0.0	10.0	10.0	89.09	70.47
F_9	1	10.0	10.0	8.1	5.0	101.37	78.66
F_{10}	23	10.0	10.0	15.0	10.0	98.01	75.33
F_{11}	17	15.0	5.0	15.0	5.0	77.88	88.18
F_{12}	11	15.0	5.0	15.0	10.0	78.60	83.62
F_{13}	26	20.0	0.0	6.0	7.5	87.37	69.67
F_{14}	24	10.0	10.0	10.7	7.8	101.07	75.65
F_{15}	4	15.0	5.0	10.6	7.2	76.23	85.41
F_{16}	27	15.0	5.0	5.0	5.0	81.74	84.63
F_{17}	7	15.0	5.0	8.0	10.0	79.98	82.61
F_{18}	2	15.0	5.0	5.0	8.5	82.83	80.27
F_{19}	15	20.0	0.0	14.2	7.5	83.62	70.42
F_{20}	10	10.0	10.0	8.8	9.5	99.73	73.90
F_{21}	20	12.6	7.4	15.0	7.5	80.31	81.29
F_{22}	21	17.5	2.5	9.7	5.0	84.08	78.72
F_{23}	19	20.0	0.0	10.0	7.7	84.21	74.93
F_{24}	16	10.0	10.0	15.0	10.0	107.42	76.89
F_{25}	12	10.0	10.0	15.0	5.0	96.35	80.97
F_{26}	22	15.0	5.0	15.0	5.0	77.10	90.07
F_{27}	25	10.0	10.0	8.1	5.0	95.14	77.12
F_{28}	6	20.0	0.0	15.0	5.0	82.84	71.52

ANOVA and coded coefficients of Full as well as reduced model for vesicle size are presented in **Table 5A-8** and **Table 5A-9**, respectively. ANOVA table for vesicle size showed insignificant quadratic effect among selected CMA. However, the linear mixture and interactive effects between P90G and P90H as well as P90G and Surfactant were found to affect vesicle size significantly. An insignificant lack-of fit showed the adequacy of the model in explaining the variation in the responses.

Table 5A-8. Analysis of variance of full and reduced quadratic model for vesicle size

Source	Full model					Reduced model (α out - 0.1)*				
	DF	Adj SS	Adj MS	F-Value	P-Value	DF	Adj SS	Adj MS	F-Value	P-Value
Model	8	2450.92	306.37	28.90	< 0.0001	4	2403.54	600.88	55.54	< 0.0001
Linear Mixture	1	1005.44	1005.44	94.83	< 0.0001	1	1005.44	1005.44	92.93	< 0.0001
AB	1	1183.26	1183.26	111.60	< 0.0001	1	1234.11	1234.11	114.07	< 0.0001
AC	1	57.38	57.38	5.41	0.0312	1	103.48	103.48	9.56	0.0051
AD	1	16.20	16.20	1.53	0.2315					
BC	1	13.23	13.23	1.25	0.2778					
BD	1	51.91	51.91	4.90	0.0394	1	40.15	40.15	3.71	0.0665
ABC	1	1.14	1.14	0.11	0.7467					
ABD	1	24.95	24.95	2.35	0.1415					
Residual	19	201.45	10.60			23	248.83	10.82		
Lack-of-Fit	14	119.82	8.56	0.52	0.8432	18	167.20	9.29	0.57	0.8274
Pure Error	5	81.63	16.33			5	81.63	16.33		
Total	27	2652.37				27	2652.37			

* Shaded rows represent insignificant model terms removed during model reduction

Table 5A-9. Coded coefficients of full as well as reduced quadratic model for vesicle size

Term	Full Model			Reduced model (α out - 0.1)*		
	Coef	SE Coef	VIF	Coef	SE Coef	VIF
A-90G	87.43	1.088	1.27	87.56	1.09	1.26
B-90H	101.41	1.037	1.25	101.18	1.03	1.22
AB	-59.83	5.664	1.54	-59.68	5.59	1.47
AC	-3.07	1.319	1.27	-3.69	1.19	1.02
AD	1.65	1.333	1.32			
BC	-1.44	1.293	1.30			
BD	2.58	1.168	1.18	2.10	1.09	1.01
ABC	-2.21	6.740	1.54			
ABD	-10.27	6.693	1.51			

* Shaded rows represent insignificant model terms removed during model reduction

Coefficients table for vesicle size showed VIF values near to 1 indicating that the predictors are not correlated and regression coefficients are well estimated. Regression equations for full and reduced models in uncoded units are presented as **Eq. 5A-2** and **Eq. 5A-3**, respectively. The positive and negative sign before each coefficients indicates a direct or inverse relationship of that model term with vesicle size.

Full model

$$R1 = 88.63A + 153.70B - 98.45AB - 0.61AC + 0.66AD + 0.92BC + 9.62BD - 1.77ABC - 16.43ABD$$

Eq. 5A-2

Reduced model

$$R1 = 94.95A + 214.15B - 238.71AB - 0.74AC + 1.68BD$$ **Eq. 5A-3**

Based on the experimental data of drug entrapment, software suggested quadratic model for both mix order as well as process order.

ANOVA and coded coefficients of Full as well as reduced quadratic model for drug entrapment are presented in **Table 5A-10** and **Table 5A-11**, respectively.

Table 5A-10. Analysis of variance of full as well as reduced quadratic model for drug entrapment

Source	Full model					Reduced model (α out - 0.1)*				
	DF	Adj SS	Adj MS	F-Value	P-Value	DF	Adj SS	Adj MS	F-Value	P-Value
Model	17	1430.06	84.12	16.91	< 0.0001	9	1385.44	153.94	29.36	< 0.0001
Linear Mixture	1	90.04	90.04	18.10	0.0017	1	90.04	90.04	17.17	0.0006
AB	1	58.56	58.56	11.77	0.0064	1	113.25	113.25	21.60	0.0002
AC	1	51.01	51.01	10.25	0.0095	1	58.93	58.93	11.24	0.0035
AD	1	3.37	3.37	0.68	0.4296					
BC	1	145.25	145.24	29.19	0.0003	1	183.34	183.34	34.97	< 0.0001
BD	1	57.37	57.37	11.53	0.0068	1	65.38	65.38	12.47	0.0024
ABC	1	18.75	18.75	3.77	0.0809	1	21.63	21.63	4.13	0.0573
ABD	1	0.03	0.03	0.01	0.9400					
ACD	1	1.71	1.71	0.34	0.5707					
BCD	1	16.75	16.75	3.37	0.0964					
AC^2	1	17.76	17.76	3.57	0.0882	1	32.39	32.39	6.18	0.0230
AD^2	1	8.66	8.66	1.74	0.2164					
BC^2	1	53.63	53.63	10.78	0.0082	1	68.73	68.73	13.11	0.0020
BD^2	1	3.35	3.35	0.67	0.4312					
ABCD	1	4.87	4.87	0.98	0.3458					
ABC^2	1	18.57	18.57	3.73	0.0822	1	29.67	29.67	5.66	0.0286
ABD^2	1	2.51	2.51	0.50	0.4936					
Residual	10	49.75	4.98			18	94.37	5.24		
Lack of Fit	5	32.12	6.42	1.82	0.2632	13	76.73	5.90	1.67	0.2972
Pure Error	5	17.63	3.53			5	17.63	3.53		
Cor Total	27	1479.81				27	1479.81			

* Shaded rows represent insignificant model terms removed during model reduction

ANOVA table for drug entrapment showed significant interaction, quadratic and linear mixture effects among selected CMA. Significant quadratic terms indicated that the relationship between these CMA and drug entrapment follow a curved line. An insignificant lack-of fit showed the adequacy of the model in explaining the variation in the responses.

Table 5A-11. Coded coefficients of full as well as reduced quadratic model for drug entrapment

Term	Full Model			Reduced model (α out - 0.1)*		
	Coef	SE Coef	VIF	Coef	SE Coef	VIF
A-90G	73.59	1.63	6.09	72.54	1.52	5.00
B-90H	75.99	1.87	8.66	77.48	1.31	4.02
AB	32.99	9.62	9.46	35.63	7.67	5.70
AC	2.93	0.91	1.30	3.09	0.92	1.25
AD	-0.81	0.99	1.54			

Term	Full Model			Reduced model (α out - 0.1)*		
	Coef	SE Coef	VIF	Coef	SE Coef	VIF
BC	5.34	0.99	1.62	5.71	0.97	1.47
BD	-2.94	0.87	1.38	-2.73	0.77	1.04
ABC	-9.75	5.02	1.82	-9.78	4.82	1.59
ABD	0.39	5.06	1.84			
ACD	-0.61	1.04	1.32			
BCD	1.89	1.03	1.40			
AC^2	-4.11	2.18	6.90	-4.70	1.89	4.95
AD^2	-2.33	1.77	4.90			
BC^2	-5.91	1.80	5.12	-6.31	1.74	4.56
BD^2	1.71	2.09	7.62			
ABCD	-5.55	5.61	1.71			
ABC^2	19.70	10.20	7.32	22.58	9.49	6.02
ABD^2	7.10	9.99	7.00			

* Shaded rows represent insignificant model terms removed during model reduction

Coefficients table for drug entrapment showed VIF values near to 1 for 2-way as well as 3-way interaction terms while it was <10 for quadratic terms indicating that the predictors are not correlated and regression coefficients are well estimated. Regression equations for full and reduced models in uncoded units are presented as **Eq. 5A-4** and **Eq. 5A-5**, respectively. The positive and negative sign before each coefficients indicates a direct or inverse relationship of that model term with drug entrapment.

Full model

$$R2 = 29.08A - 195.99B + 642.71AB + 4.24AC + 5.76AD + 33.84BC + 5.53BD - 57.51ABC - 49.75ABD - 0.05ACD + 1.24BCD - 0.16AC^2 - 0.37AD^2 - 1.88BC^2 - 1.35BD^2 - 1.78ABCD + 3.15ABC^2 + 4.54ABD^2$$

Eq. 5A-4

Reduced model

$$R2 = 47.54A - 240.63B + 582.11AB + 4.38AC + 48.05BC - 2.18BD - 80.09ABC - 0.19AC^2 - 2.12BC^2 + 3.61ABC^2$$

Eq. 5A-5

Model summary for both CQA is presented in **Table 5A-12**. A low SD value and high R^2 value indicated a better prediction of responses by the model. Predicted R^2 was found to be in good agreement with other R^2 further supporting the prediction potential of the model.

Table 5A-12. Summary of full as well as reduced quadratic-linear model for both CQA

Responses	Full model				Reduced model (α out - 0.1)			
	SD	R-sq	R-sq (adj)	R-sq (pred)	SD	R-sq	R-sq (adj)	R-sq (pred)
Vesicle size (nm)	3.26	92.40	89.21	83.65	3.29	90.62	88.99	85.84
Drug entrapment (%)	2.23	96.64	90.92	63.56	2.29	93.62	90.43	84.50

Four different residual plots viz., normal plot of residual, residual versus ascending predicted response values, residual versus experimental run order and predicted versus actual were generated for both CQA and presented in **Fig. 5A-4** and **Fig. 5A-5**. In normal plot, residuals were appeared to follow a straight line indicating that the data was normally distributed. Random scattering without any megaphone pattern in residual versus predicted plot validated the assumption of constant variance. Similarly, random scattering without any pattern in residual versus run plot validated the absence of lurking variables. In predicted versus actual plot, data points were evenly split by the 45-degree line indicating easy prediction of values by the model [6].

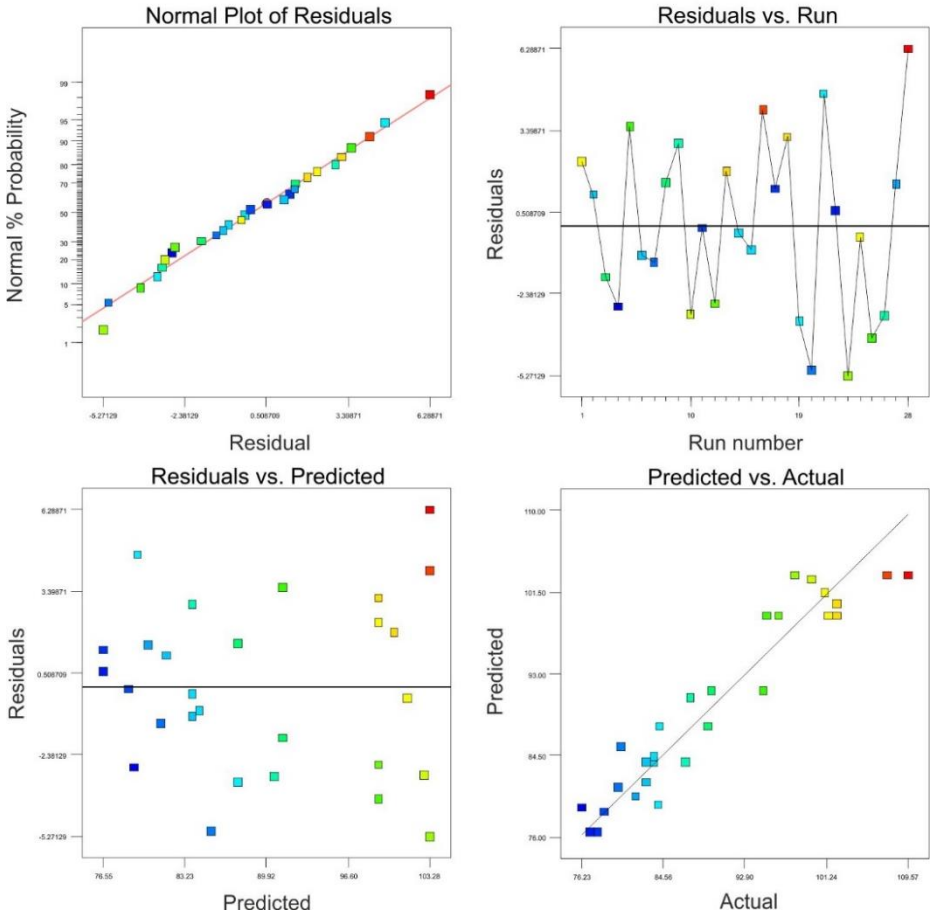

Fig. 5A-4. Residual plots for vesicle size

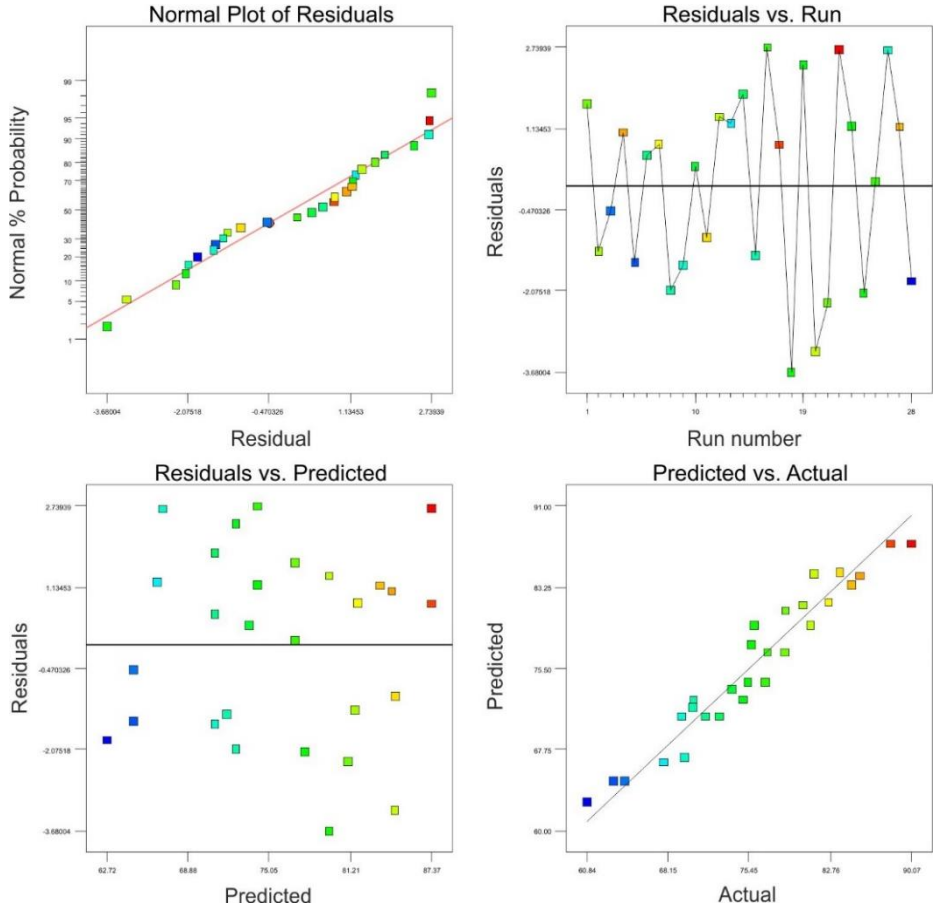

Fig. 5A-5. Residual plots for drug entrapment

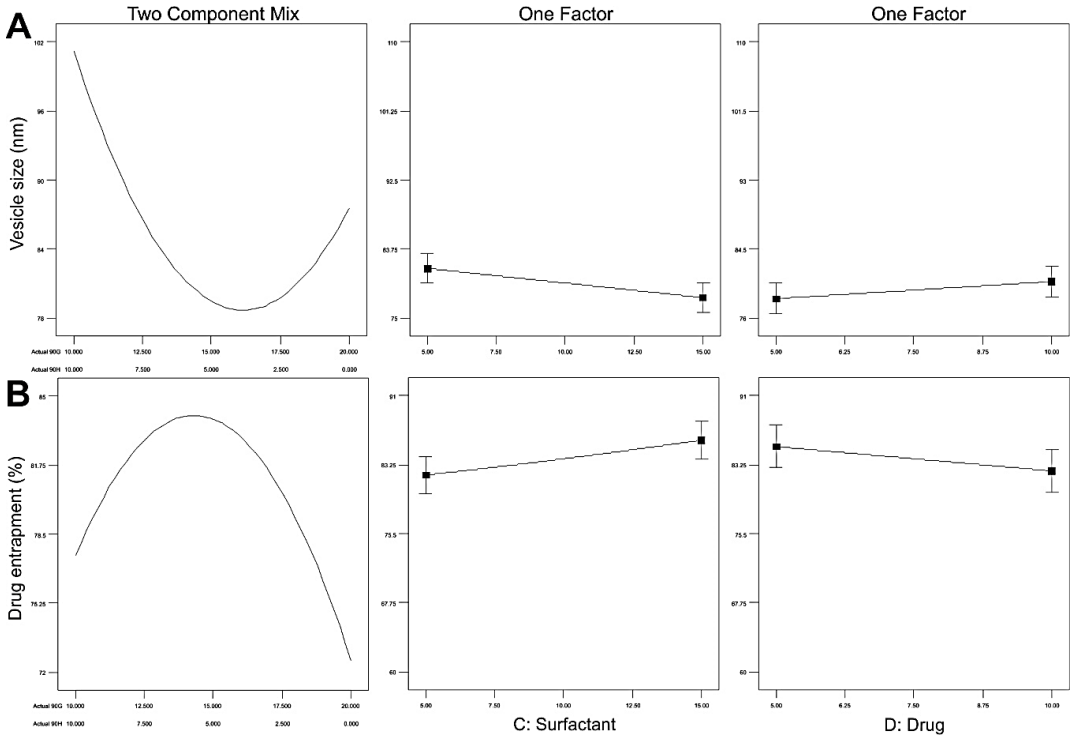

Fig. 5A-6. Main effect plots of reduced model for A. vesicle size and B. drug entrapment

The main effect plots for both CQA are presented in **Fig. 5A-6**. These graphs provided a better depiction of how the individual CMA influence respective CQA and found in-line with the ANOVA results. Contour and response surface plots are presented in **Fig. 5A-7** and **Fig. 5A-8**, respectively. These graphs were used to depict how the CQA is related to respective CMA while keeping other CMA at constant levels.

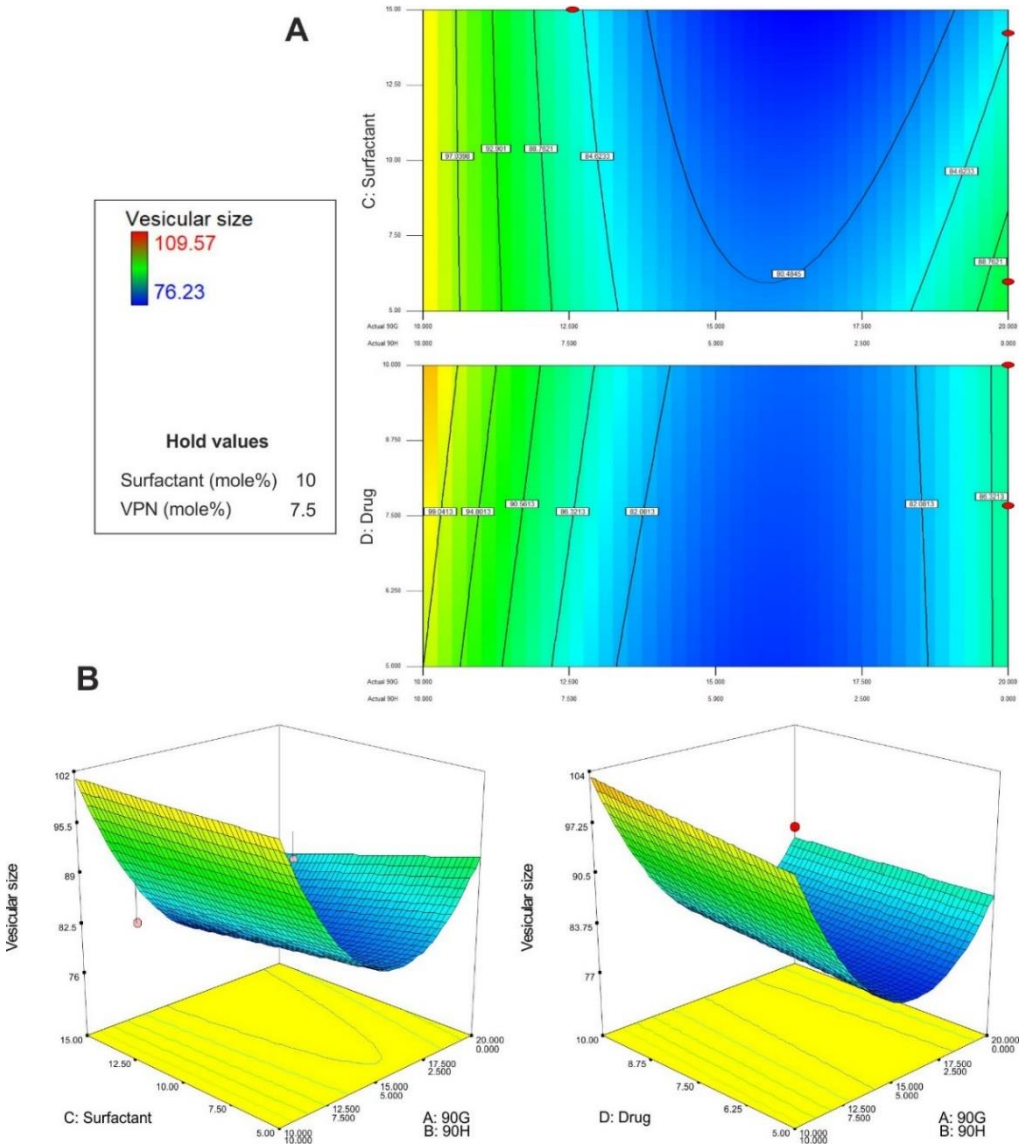

Fig. 5A-7. Contour and response surface plots of reduced model for vesicle size

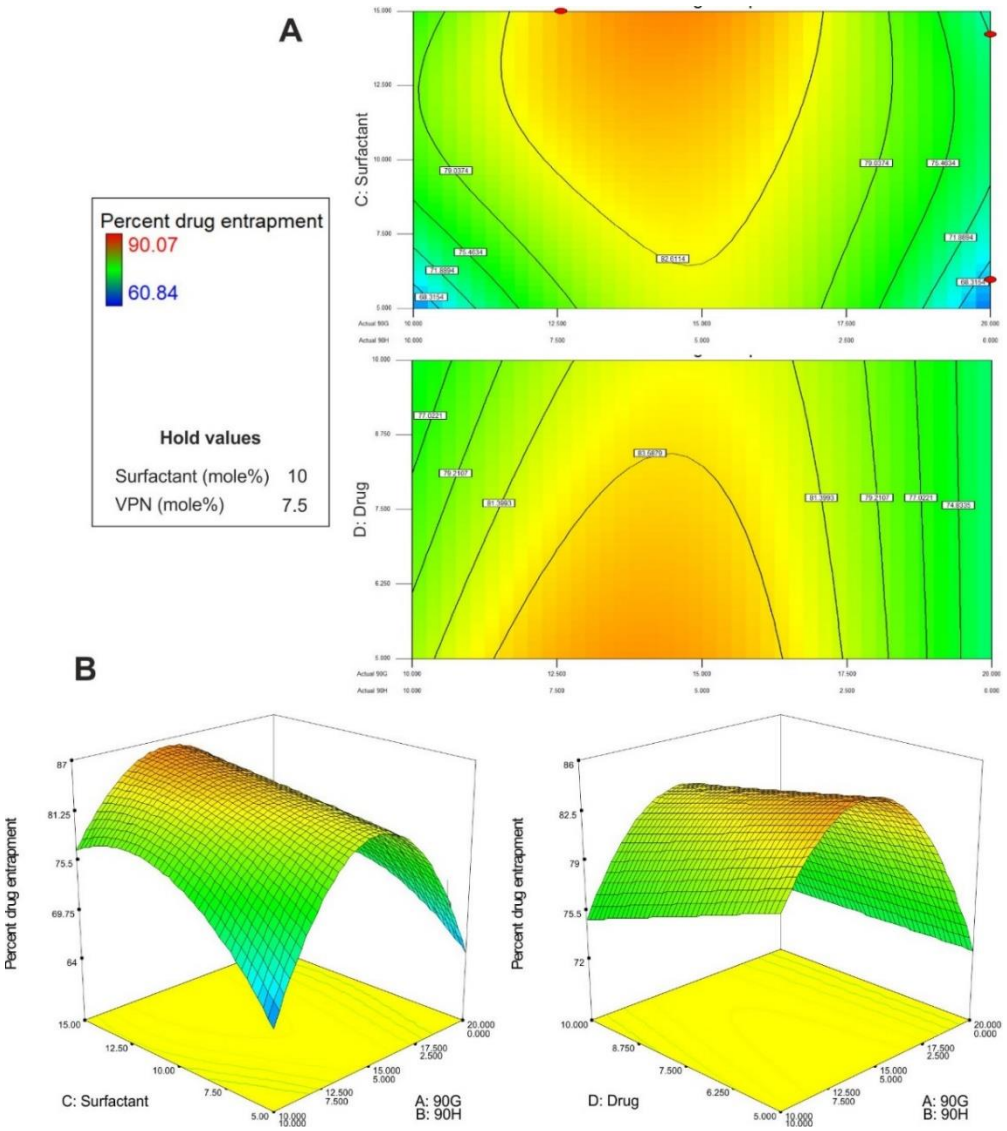

Fig. 5A-8. Contour and response surface plots of reduced model for drug entrapment

Numerical optimization was performed by the software for defined optimization criteria as presented in **Table 5A-13**. The software was programmed to provide the optimization solution with minimum vesicle size and maximum drug entrapment while keeping all the CMA within experimental range.

Table 5A-13. Criteria for optimization of VPN-UDL

Constraints name	Goal	Lower	Upper	Weight	Importance
P90G	in range	10	20	1	3
P90H	in range	0	10	1	3
Surfactant	in range	5	15	1	3
Drug	in range	5	10	1	3
Vesicle size	minimize	76.23	109.57	1	3
Percent drug entrapment	maximize	60.84	90.07	1	3

Overlaid contour plots for both CQA were generated (**Fig. 5A-9**) to observe the design space (yellow area in graph) using the defined criteria shown in above table.

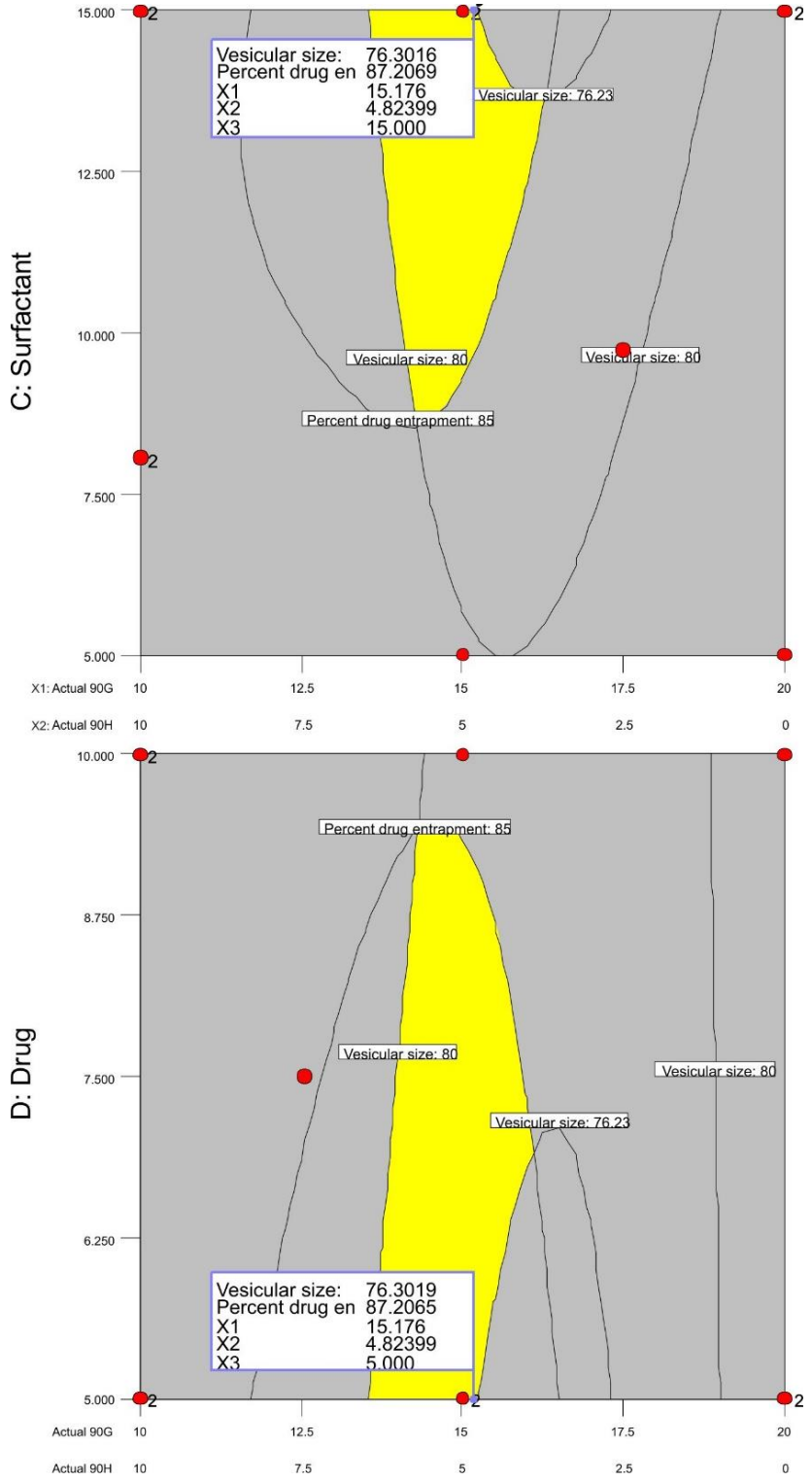

Fig. 5A-9. Overlay contour plots of reduced quadratic model showing design space

Further, the optimization solution ramp and desirability plot are presented in **Fig. 5A-10** and **Fig. 5A-11**.

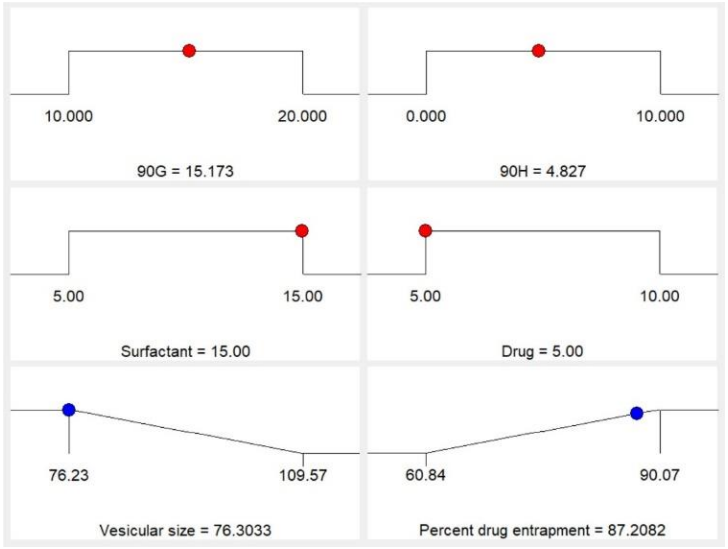

Fig. 5A-10. Optimization solution ramp for defined desirability criteria

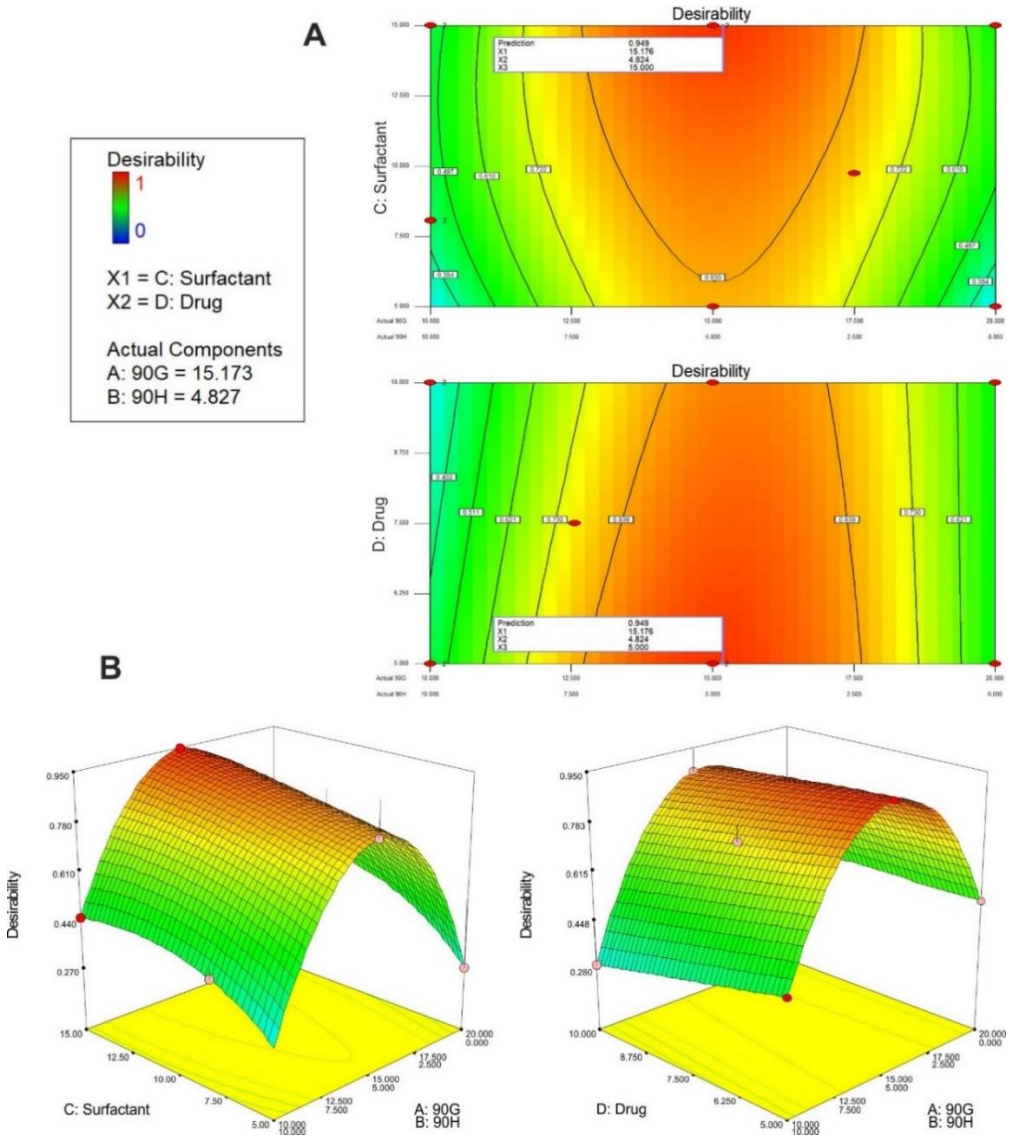

Fig. 5A-11. A) Contour and B) Response surface plot of composite desirability for both CQA

The optimization solution ramp and desirability plot showed a composite desirability of 0.949 for the solution provided by the software. The setting of this optimization solution is also presented in Table 5A-14 along with the 95% confidence as well as 95% prediction intervals. Three batches with optimized levels were prepared for verification trials and the values of both CQA are presented in Table 5A-15.

Table 5A-14. Optimization solution

Multiple Response Prediction

Variable	Setting
P90G (mM)	15.17
P90H (mM)	4.83
Surfactant (mole%)	15
Drug (mole%)	5

Responses	Fit	SE Fit	95% Confidence interval		95% Prediction interval	
			Lower	Upper	Lower	Upper
Vesicle size (nm)	76.30	1.35	73.51	79.09	68.95	83.66
Drug entrapment (%)	87.21	1.24	84.61	89.81	81.74	92.68

Table 5A-15. Results of verification trials

Responses	95% Prediction interval		Results			
	Lower	Upper	Batch-1	Batch-2	Batch-3	Average
Vesicle size (nm)	68.95	83.66	77.05	76.08	73.82	**75.65**
Drug entrapment (%)	81.74	92.68	85.96	88.24	88.13	**87.44**

The average values of both CQA were found to fall within 95% confidence interval and thus indicated the validity of the model.

5A.3.2 *In vitro* characterization of optimized VPN-UDL

5A.3.2.1 Shape and surface morphology

Transmission electron microscopy of optimized VPN-UDL was performed and the image is represented as Fig. 5A-12. The image showed spherical shape with smooth surface of liposomes. The size of liposomes seen in the image was found in-line with the results of vesicle size data obtained from Malvern zetasizer (Fig. 5A-13).

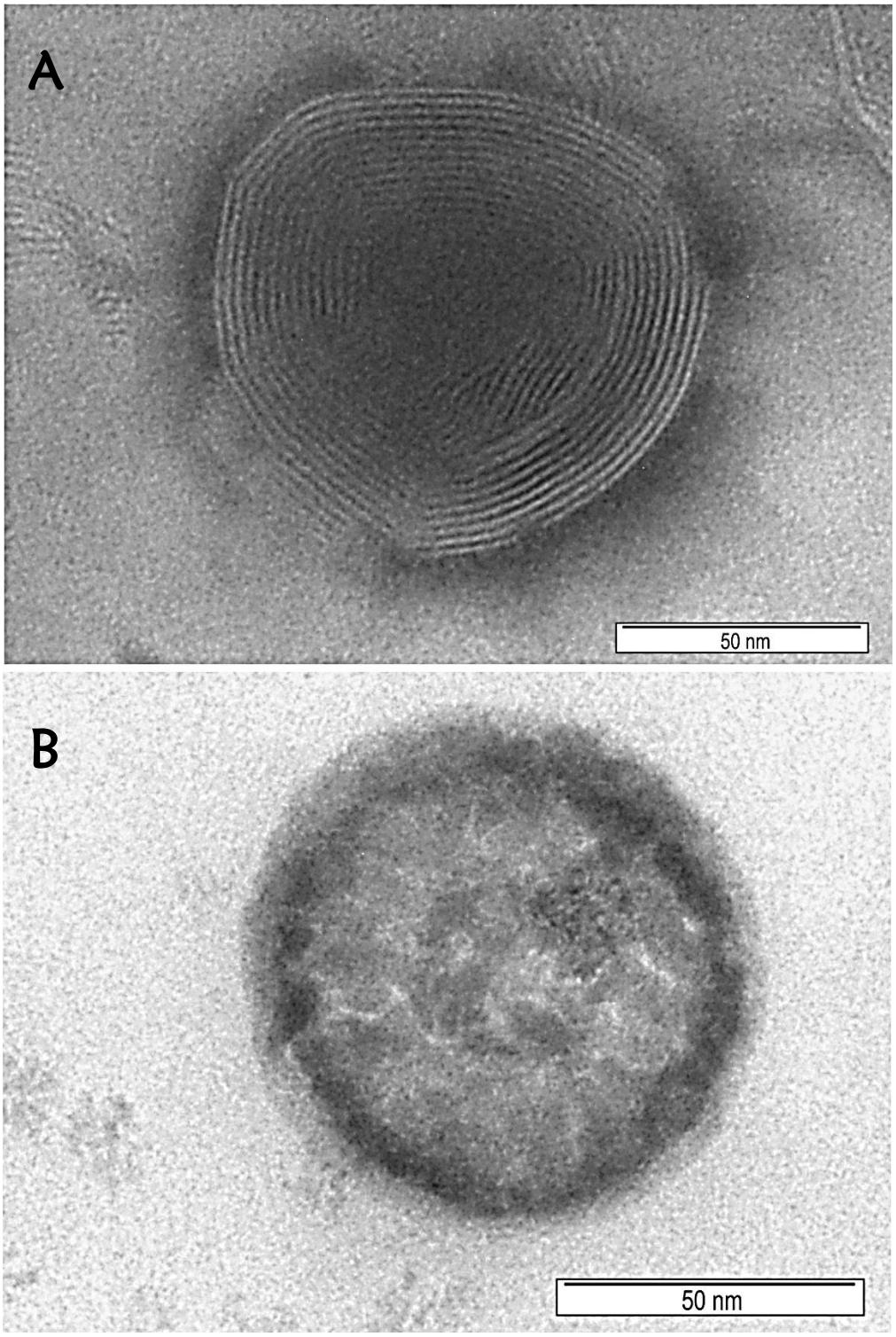

Fig. 5A-12. Transmission electron microscopic images showing (A) multi-lamellae and (B) surface of optimized VPN UDL

System

Temperature (°C):	25.0	**Duration Used (s):**	80
Count Rate (kcps):	162.6	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	6

Results

			Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm):	77.05	**Peak 1:**	77.10	100.0	82.40
PdI:	0.216	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.960	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

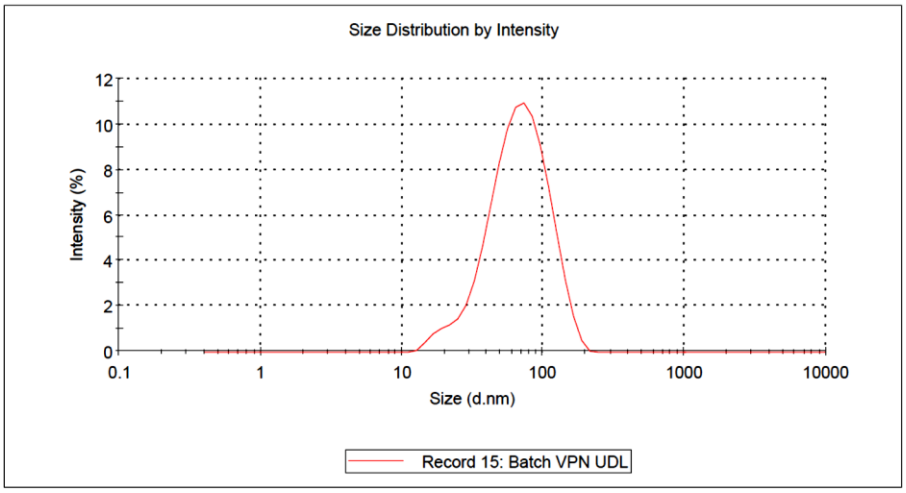

Fig. 5A-13. Size distribution graph of optimized VPN-UDL

System

Temperature (°C):	25.0	**Zeta Runs:**	12
Count Rate (kcps):	127.6	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	10

Results

			Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	-31.7	**Peak 1:**	-31.7	100.0	6.55
Zeta Deviation (mV):	6.55	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.170	**Peak 3:**	0.00	0.0	0.00
Result quality :	Good				

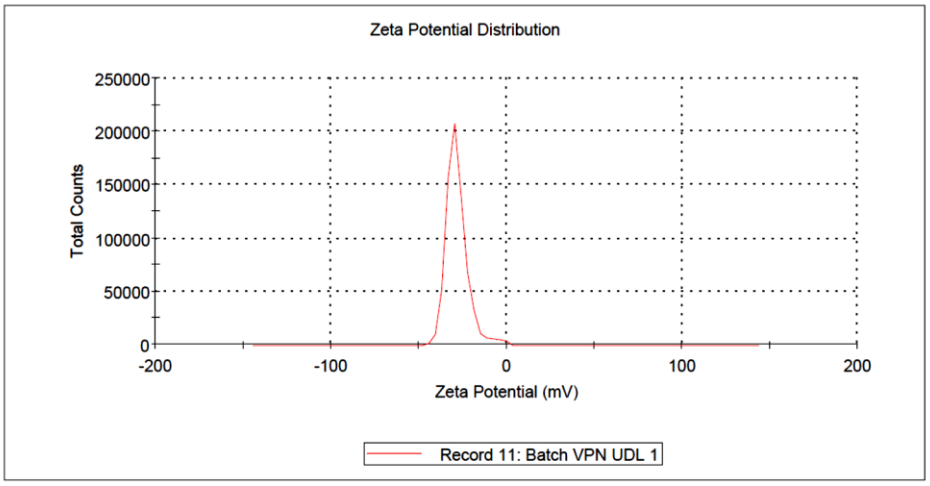

Fig. 5A-14. Zeta potential graph of optimized VPN-UDL

5A.3.2.2 Zeta potential

The zeta potential graph of optimized VPN-UDL (**Fig. 5A-14**) showed a net negative charge on liposome surface with a zeta potential value of -31.7 mV. The charge was found sufficient enough to keep the particles dispersed via repulsive forces [7, 8].

5A.3.2.3 In-vitro drug release study

In vitro drug release from VPN UDL was evaluated and the cumulative percent drug release at different time points are summarized in **Table 5A-16** as well as illustrated in **Fig. 5A-15**. In order to ensure that the presence of VPN in release media directly reflects its release from nanocarriers, the permeation of released VPN across dialysis membrane should not be rate limiting. Hence, data for VPN solution was also generated which showed >90% drug release within 2 hours indicating non-barrier nature of dialysis membrane for dissolved VPN. Release data of VPN UDL showed >50 % VPN release in first 8 hours and > 80 % release in 24 hours indicating the controlled release behavior of UDL.

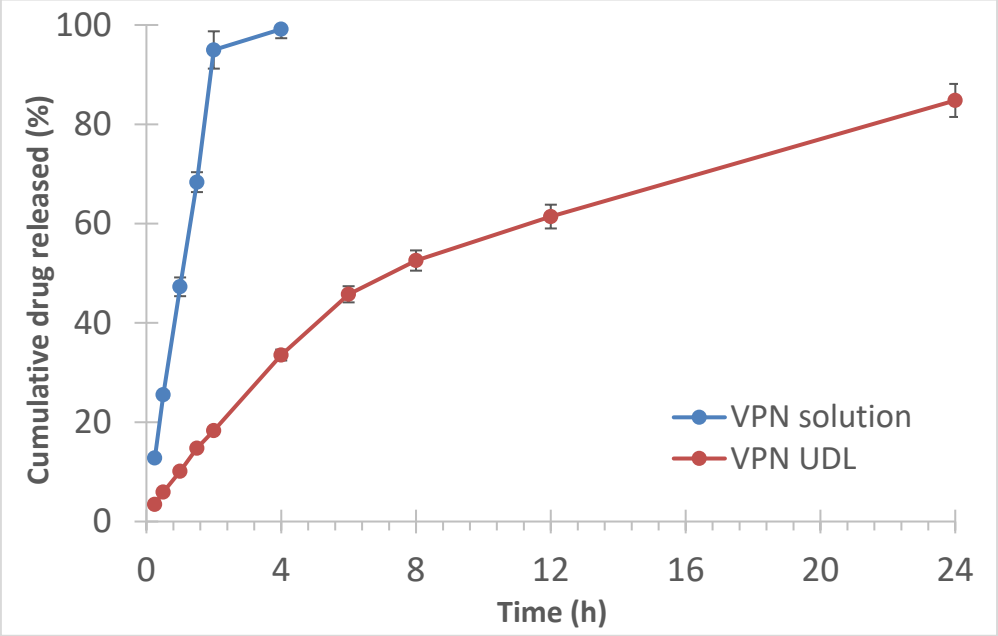

Fig. 5A-15. Cumulative percent of VPN released *in vitro* versus time curve for UDL

Table 5A-16. *In vitro* release profile of VPN from its solution and UDL

Time (h)	Cumulative percent drug released	
	VPN Solution*	VPN UDL*
0.25	12.78 ± 0.35	03.46 ± 0.09
0.5	25.54 ± 0.69	05.94 ± 0.13

Time (h)	Cumulative percent drug released	
	VPN Solution*	VPN UDL*
1	47.27 ± 1.89	10.11 ± 0.38
1.5	68.35 ± 2.01	14.77 ± 0.28
2	94.97 ± 3.76	18.29 ± 0.35
4	99.16 ± 1.82	33.53 ± 1.10
6	-	45.76 ± 1.63
8	-	52.56 ± 2.04
12	-	61.42 ± 2.39
24	-	84.80 ± 3.33

* Result represented as mean ± SD

The result of various mathematical models, applied to understand the VPN release kinetics from UDL, are presented in Table 5A-17.

Table 5A-17. Various mathematical models and their correlation coefficient values

Mathematical models	Graph description (Y-axis versus X-axis)	VPN UDL	
		R^2	n
Zero order	Cumulative amount/percent of drug released *versus* time	0.943	-
First order	Log cumulative percent drug remaining *versus* time	-0.997	-
Higuchi	Cumulative percent drug released *versus* square root of time	0.994	-
Hixon Crowell	Cube root of percent drug remaining *versus* time	-0.986	-
Korsmeyer Peppas	Log cumulative percent drug released *versus* log time	0.994	0.78

The R^2 values for Higuchi as well as first order model was found higher suggesting a diffusion controlled system where release rate was dependent on remaining drug concentration within the nanocarrier.

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Contents

5B.1. INTRODUCTION

With an objective to achieve therapeutic plasma levels of NPT via transdermal route, ultra-deformable liposomes (UDLs) were chosen for their capability to flow through the narrow intercellular pores and carry the active compound into deeper layers of the skin [1]. Out of several available methods for preparation, ethanol injection method was used for NPT [2]. A systematic Quality-by-design (QbD) approach employing statistical design of experiments was utilized to exhaustively evaluate the impact of material attributes and process parameters on the critical formulation attributes [3].

5B.2. MATERIALS & METHODS

5B.2.1 Materials

Noopept (NPT) was purchased from Nootrico SA, United States. Phospholipon 90G (Soya phosphatidylcholine, P90G) and Phospholipon 90H (hydrogenated Soya phosphatidylcholine, P90H) were kindly gifted by Lipoid GmBH, Germany. Sodium deoxycholate (SDC) was purchased

from S.D. Fine Chemicals, Mumbai, India. Dialysis bags (MWCO, 12 kD) were purchased from HiMedia Labs Pvt. Ltd., Mumbai, India. Double distilled water was prepared in lab, filtered through 0.2μ membrane filter in glass bottle and consumed within a maximum of 7 days.

5B.2.2 Preparation of Noopept loaded ultradeformable liposomes (NPT UDL)

Noopept loaded UDL was prepared using ethanol injection method. Briefly, the NPT, P90G, P90H and SDC were dissolved in ethanol. A 5 ml of prefiltered distilled water was taken in a round bottom flask and continuously stirred on a magnetic stirrer at room temperature. Then organic phase was slowly added into aqueous phase using a 1ml syringe. The stirring was continued for next 15 minutes at room temperature and later, ethanol was evaporated using rotary evaporator. The UDL dispersion was centrifuged for 10 minutes at 5000 rpm and 15°C for the sedimentation of free drug. The supernatant liposomal dispersion was carefully separated without disturbing the free drug pellet at the bottom. The separated liposomal dispersion was stored in glass vials at room temperature till further analysis.

5B.2.2.1 Establishing Quality target product profile and Critical Quality Attributes

Based on the scientific, therapeutic, industrial and regulatory aspects, quality target product profile (QTPP) for NPT loaded ultradeformable liposomes were established. Further, based on the prior knowledge, literature review and experiment trials, two response variables viz., vesicular size and drug entrapment were selected as critical quality attributes (CQA).

5B.2.2.2 Identification of Independent variables (factors) and qualitative risk assessment

Ishikawa diagram was used to demonstrate all the probable variables associated with development of NPT loaded UDL by ethanol injection method. These factors were qualitatively categorized as 'low,

medium and high risk' based on their impact on CQA as described in **Table 5B-1**.

Table 5B-1. Quality risk assessment criteria

Low Risk	Factors with wide range of acceptability. No investigation required
Medium Risk	Acceptable risk. No adverse effect on product quality on small changes.
High Risk	Unacceptable risk. Acceptable range need to be investigated

Factors with low and medium risk were controlled by assigning constant levels based on literatures and preliminary trials.

5B.2.2.3 Quantitative risk assessment: Screening design

Factors with high risk were screened using 2-level fractional factorial design to statistically identify the critical factors and use them in main design to determine the control ranges (design space). Screening design was also utilized to assign constant levels of other non-critical factors. Minitab® 17.1.0 was used to generate a randomized design matrix based on which experimental batches were prepared and evaluated for CQA. Software based Pareto charts were utilized to determine critical factors while the main effect charts were utilized to decide the optimum levels of non-critical factors. Methods used for estimation of CQA are as follows

5B.2.2.3.1 Vesicular size and size distribution

Liposomal dispersions were diluted ten times with pre-filtered distilled water, transferred to disposable sizing cuvette and analyzed by dynamic light scattering (DLS) using Nano-ZS Zetasizer, Malvern Instruments Ltd., UK for vesicular size (VS) and poly-dispersity index (PDI). The instrument analyzes angular scattering of a laser beam during its passage through the dispersed liposomal sample and use the Mie theory of light scattering to calculate the mean diameter of liposomes.

5B.2.2.3.2 Drug entrapment

Samples from liposomal dispersions (0.1 ml) were dissolved and suitably diluted in acetonitrile and analyzed using HPLC method as described earlier in chapter 3. Drug entrapment (%) was then calculated using **Eq. 5B-1**,

$$\text{Drug entrapment}\ (\%) = \frac{\text{Entrapped drug (mg)}}{\text{Total drug taken (mg)}} \times 100 \qquad \textbf{Eq. 5B-1}$$

5B.2.2.4 Formulation optimization by combined D-optimal response surface design

Combined D-optimal response surface design was applied to exhaustively investigate the relationship between critical factors and CQA with less number of experimental batches while handling mixture components and other numeric factors simultaneously [4]. Design Expert® 7.0.0 was used for generating the randomized design matrix and statistical evaluation of experimental data to achieve optimization solution and creating the design space. Suitability of model suggested by the software and identification of significant model terms were decided based on analysis of variance followed by F-test. Insignificant model terms were later removed to simplify the mathematical equations for calculation of CQA. The relationship between critical factors and CQA was explored using contour and 3-D response surface plots. Desirability criteria was defined based on QTPP and design space was created to obtain final optimized batch. Three batches were prepared with optimized composition for model verification.

5B.2.3 *In vitro* characterization of optimized NPT UDL

5B.2.3.1 Shape and surface morphology

The NPT UDL (NPT loaded UDL with optimized composition) were evaluated for shape and surface characteristics using transmission electron microscopy. Dispersion was spread on a carbon-coated grid, excess solution was removed and the grid was dried under infrared lamp. It was negatively stained with 2% phosphotungstic acid (PTA) and again dried under Infrared lamp. Transmission electron microscope (CM 200, Philips, Netherlands) with operating voltage range of 20-200 kV was used to visualize liposomes at suitable enlargement with an accelerating voltage of 20 kV.

5B.2.3.2 Zeta potential

NPT UDL dispersion was diluted ten times with pre-filtered distilled water, transferred to disposable folded capillary cells and analyzed for zeta potential (ZP) using Nano-ZS zetasizer. The instrument utilizes Smoluchowski equation that calculates zeta potential based on amount of doppler shift occur due to electrophoretic mobility of colloidal particles in response to the electric field applied to the dispersion.

5B.2.3.3 In-vitro drug release study

The *in-vitro* drug release from optimized NPT UDL was evaluated using a Franz-type diffusion cell with an effective surface area of 3.14 cm^2 and a receptor chamber volume of 15 ml. Pre-activated dialysis membrane (MWCO, 12 kD) was mounted, as a permeation barrier, between donor and receptor chambers of diffusion cell. The receptor chamber was filled with a mixture of ethanol and double distilled water (ratio 3:7) as a diffusion media and allowed to equilibrate for half an hour. The optimized batches containing 1 mg of drug were transferred to donor chambers of diffusion cells. The diffusion medium was continuously stirred using a magnetic stirrer. 1 mL sample was withdrawn from sampling arm of diffusion cell at each time point up to 24 hours and equal volume of fresh diffusion media was added to maintain total receptor volume. Quantitative estimation of drug was performed using HPLC method at 258 nm detection wavelength as described earlier in chapter 3. The kinetics of drug release was then evaluated by fitting the data in various mathematical models and comparing their regression coefficient (R^2) values [5].

5B.3. RESULTS & DISCUSSION

5B.3.1 Preparation and optimization of NPT loaded ultradeformable liposomes

5B.3.1.1 Establishing QTPP and CQA

Various QTPP elements and their targets were defined and presented with justification in Table 5B-2.

Table 5B-2. QTPP elements with justification for NPT loaded UDL

QTPP element		Target	Justification
Route of administration		Transdermal	Avoid first pass metabolism and achieve prolonged action
Dosage form		Ultradeformable liposome	Better skin permeability and controlled drug release
Formulation quality attributes	Vesicular size#	Minimize (~100 nm)	To ensure better permeation and drug release
	Polydispersity Index	Minimize (< 0.3)	To ensure uniformity of size and related characteristics.
	Zeta potential	> ±30 mV	To ensure stability of the dispersion
	Surface characteristics	Spherical, smooth	To ensure better permeation
	Drug entrapment#	Maximize	To minimize drug wastage for cost-effectiveness
	Shape	Ultradeformable	For better skin permeation
	In vitro Drug release behavior	Prolonged for 24 hours	To ensure controlled drug release for desired duration
Ex vivo permeability		Better transdermal flux	To ensure PK/ PD comparable to marketed formulations
Stability		NLT 1 months	To ensure stability till incorporation in final dosage form
Safety		Non-toxic & Non-irritant to skin	To ensure safety of the final formulation
Pharmacokinetics		Similar or better than oral suspension	For bioequivalence requirement
Pharmacodynamics			To demonstrate therapeutic efficacy

Critical quality attributes

Vesicular size and drug entrapment were identified as critical in governing the product quality and need to be within known limits to attain the pre-defined QTPP. Thus, these three characteristics were selected as CQA.

5B.3.1.2 Identification and qualitative assessment of Independent variables (factors)

All the probable variables associated with development of NPT loaded UDL by ethanol injection method were identified during the brainstorming sessions and categorized in to Material, Process, Equipment, Personnel and Environment. An ishikawa diagram illustrating

the cause and effect relationship among identified variables and CQA was constructed (**Fig. 5B-1**).

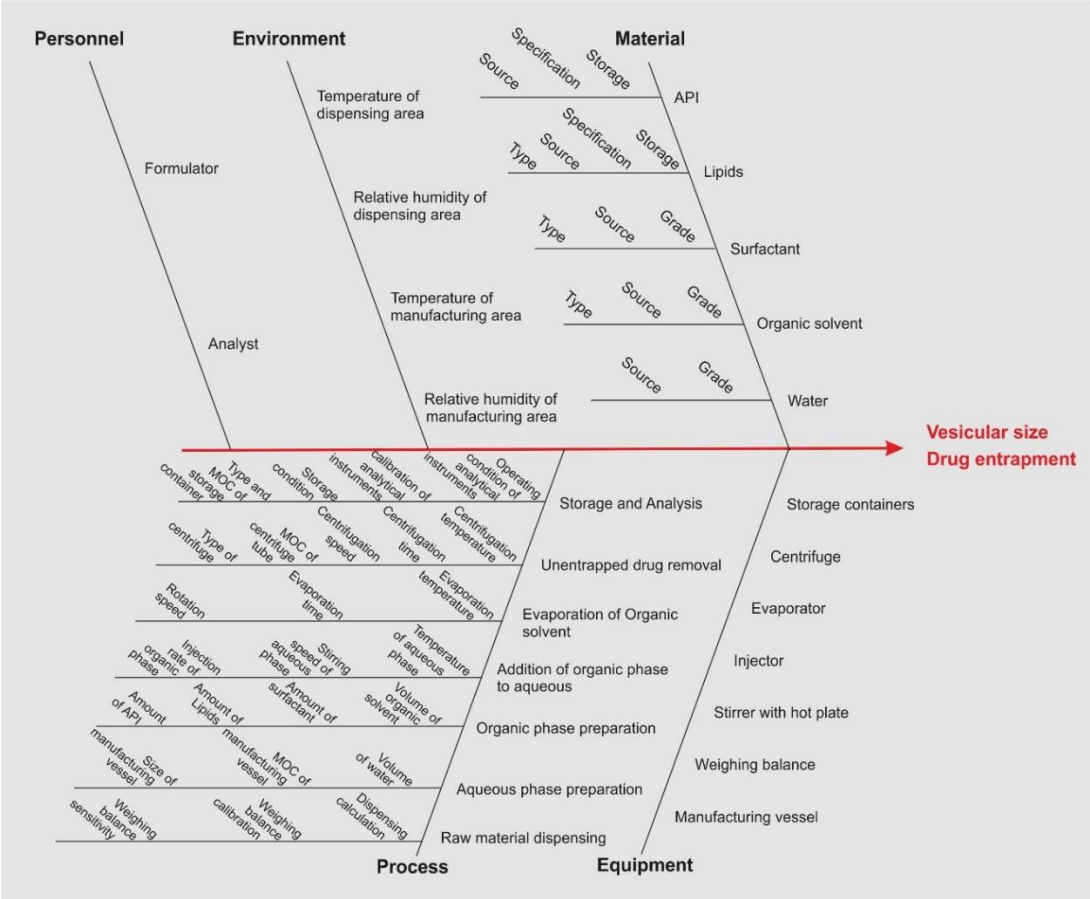

Fig. 5B-1. Ishikawa diagram showing probable variables that may influence the CQA

5B.3.1.3 Qualitative risk assessment

The risk associated with all the identified factors were evaluated based on the predefined criteria (**Table 5B-1**) and the result is presented in **Table 5B-3**. Factors with low and medium risk were assigned with the best available constant levels based on literature and preliminary trials to ensure no or negligible impact of these factors on CQA. These constant levels are also listed in **Table 5B-3**.

Table 5B-3. Qualitative risk assessment of independent variables

Factors	Process step	Impact on CQA	Constant levels
Source and specifications of API	Raw material Selection and Storage	Low risk	Authentic source with COA
Storage condition of API		Low risk	Stored at recommended condition
Type of Lipid with low Tg		Low risk	Phospholipon 90G

Factors	Process step	Impact on CQA	Constant levels
Type of Lipid with high Tg		Low risk	Phospholipon 90H
Source and specifications of lipids		Low risk	Authentic source with COA
Storage condition of Lipids		Low risk	Stored at recommended condition
Type of Surfactant		Medium risk	Sodium deoxycholate
Source and specifications of Surfactant		Low risk	Authentic source
Storage condition of Surfactant		Low risk	Stored at recommended condition
Type of Organic solvent		Medium risk	Ethanol
Source and specifications of Organic solvent		Low risk	Authentic source
Source of water		Low risk	In house
Grade of water		Low risk	Filtered (0.2µ) Double distilled
Weighing balance sensitivity	Dispensing	Low risk	0.1 mg
Weighing balance calibration		Low risk	Calibrated
Temperature and RH of Dispensing Area		Low risk	25±3°C, Ambient RH
Dispensing calculations		Low risk	Calculated using excel and verified
Type, Size and Material of Construction (MOC)	Manufacturing Vessel	Low risk	50 mL round bottom flask of class A borosilicate glass
Temperature and Relative humidity	Manufacturing Area	Low risk	25±3°C, Ambient RH
Volume of Water	Aqueous phase preparation	Low risk	5 mL
Amount of API	Organic phase preparation	High risk	To be optimized
Amount of Lipids		High risk	To be optimized
Amount of Surfactant		High risk	To be optimized
Volume of Organic solvent		High risk	To be optimized
Calibration of Injector and stirring equipment	Addition of Organic phase to aqueous	Low risk	Calibrated
Injection Rate of Organic phase		High risk	To be optimized
Stirring speed of Aqueous phase		High risk	To be optimized

Factors	Process step	Impact on CQA	Constant levels
Temperature of Aqueous phase		Low risk	60 °C
Evaporation time	Evaporation of Organic solvent	Low risk	1 hours
Evaporation temperature		Low risk	60 °C
Stirring speed during evaporation		Low risk	100 rpm
Type of Centrifuge	Unentrapped drug removal	Low risk	Cooling centrifuge
Type and MOC of Centrifuge tube		Low risk	15 mL conical-bottom glass tube with screw cap
Centrifugation speed		Medium risk	5000 rpm
Centrifugation time		Low risk	10 minutes
Centrifugation temperature		Low risk	15°C
Type and MOC of Storage container	Storage and Analysis	Low risk	20 mL flat-bottom glass vial with screw cap
Storage condition		Medium risk	Room Temperature
Calibration of Analytical Instruments		Low risk	Calibrated
Methods used of Analysis		Low risk	Validated
Formulator	Personnel	Low risk	Common for all experiments and analysis
Analyst		Low risk	

Factors with high risk were carried forward for quantitative risk assessment.

5B.3.1.4 Quantitative risk assessment: Screening Design

Factors with high risk were statistically assessed by 2-level fractional factorial screening design. The low (-1) and high (+1) levels of all the independent variables were decided based on literatures as well as preliminary trials and are listed in **Table 5B-4**.

Table 5B-4. Various material attributes and process parameters along with their levels for screening by fractional factorial design

Independent variables		Unit	Levels	
			-1	+1
A:	Amount of Lipid-1 (Phospholipon 90G)	mM	10	15
B:	Amount of Lipid-2 (Phospholipon 90H)	mM	5	10
C:	Amount of Drug	Mole %	5	10
D:	Surfactant concentration	Mole %	5	15
E:	Rate of organic phase addition	mL/min	0.5	1.0
F:	Stirring speed	rpm	500	1000
G:	Volume of organic solvent	mL	1	2

The randomized design matrix of 17 experimental batches (including one center point) was generated using Minitab® 17.1.0 statistical software and presented in **Table 5B-5**.

Table 5B-5. Randomized batch matrix and resulting CQA for screening design

Batch no.	Run order	Independent Variables							CQA	
		A	B	C	D	E	F	G	Vesicular size (nm)	Drug Entrapment (%)
S_1	02	-1	-1	-1	-1	-1	-1	-1	84.90	75.71
S_2	03	+1	-1	-1	-1	+1	-1	+1	75.53	58.47
S_3	10	-1	+1	-1	-1	+1	+1	-1	94.25	79.02
S_4	16	+1	+1	-1	-1	-1	+1	+1	88.25	75.14
S_5	11	-1	-1	+1	-1	+1	+1	+1	97.10	86.29
S_6	13	+1	-1	+1	-1	-1	+1	-1	82.22	65.23
S_7	15	-1	+1	+1	-1	-1	-1	+1	99.60	88.15
S_8	14	+1	+1	+1	-1	+1	-1	-1	89.68	79.12
S_9	09	-1	-1	-1	+1	-1	+1	+1	80.72	65.97
S_{10}	06	+1	-1	-1	+1	+1	+1	-1	73.04	57.05
S_{11}	08	-1	+1	-1	+1	+1	-1	+1	91.53	78.67
S_{12}	04	+1	+1	-1	+1	-1	-1	-1	82.26	73.63
S_{13}	01	-1	-1	+1	+1	+1	-1	-1	84.19	73.81
S_{14}	07	+1	-1	+1	+1	-1	-1	+1	82.08	65.04
S_{15}	12	-1	+1	+1	+1	-1	+1	-1	93.66	82.96
S_{16}	17	+1	+1	+1	+1	+1	+1	+1	82.62	69.70
S_{17}	05	0	0	0	0	0	0	0	84.44	71.13

The data were statistically processed by Minitab software to generate pareto, normal and main effect plots for both CQA considering $P < 0.05$ as a level of significance.

Pareto and normal charts (**Fig. 5B-2**) clearly showed that amount of lipids, drug and surfactant had a significant effect on vesicular size of resulting liposomes. Similarly, amount of lipids showed significant impact

on drug entrapment. Owing to these observations, amount of lipids, drug and surfactant were selected as CMA for the final optimization step.

The influence of injection rate, stirring speed and organic solvent volume was found insignificant on both CQA. Hence, main effect plots were utilized to decide the constant level of these factors for final optimization step. Considering the positive impact on vesicular size and/or drug entrapment (**Fig. 5B-3**), lower levels of all three factors (injection rate, 0.5 mL/min; stirring speed, 500 rpm and organic solvent volume, 1 mL) were chosen.

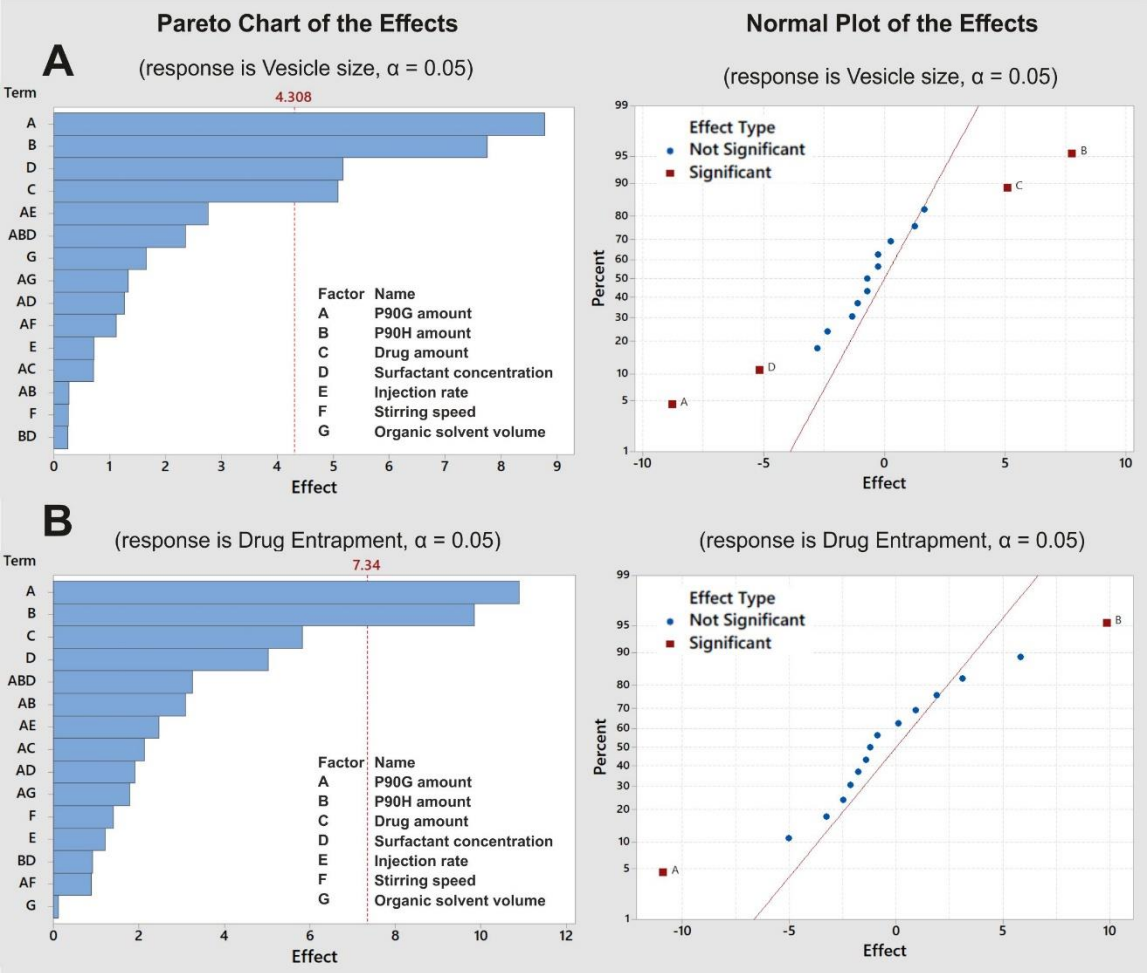

Fig. 5B-2. Pareto and Normal plots for A. Vesicle size and B. Drug entrapment

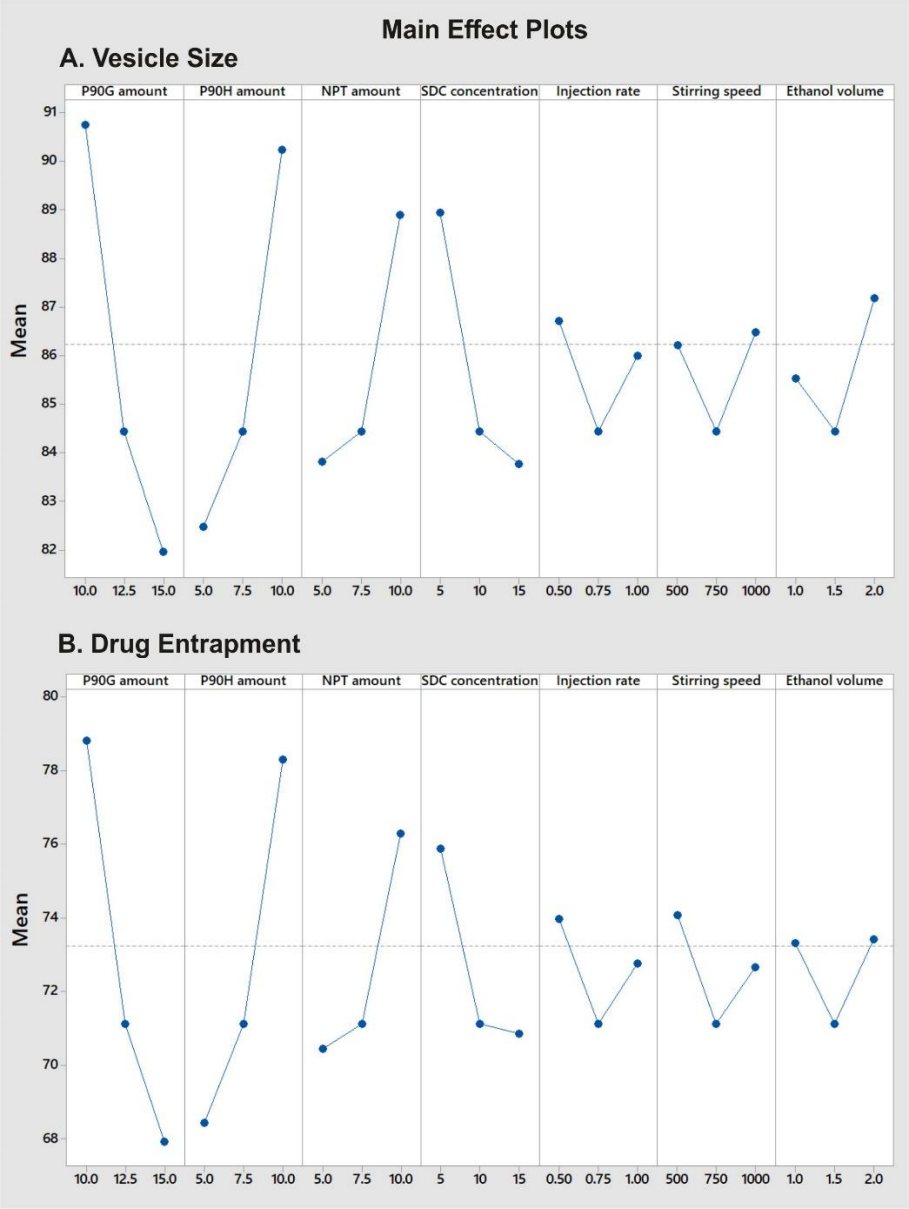

Fig. 5B-3. Main effect plots for A. Vesicular size, and B. Drug entrapment

5B.3.1.5 Formulation optimization by combined D-optimal response surface design

Based on the results of screening design, four CMA were identified and their relationship with CQA were exhaustively investigated using combined D-optimal response surface design. Combined D-optimal design was selected as it allowed the evaluation of lipid mixture together with other numerical variables viz., drug amount and surfactant concentration. The low (-1), medium (0) and high (+1) levels of all four CMA are listed in **Table 5B-6**. Amount of P90G and P90H in lipid mixture was varied in such a way to keep the total lipid level at 20 mM.

Table 5B-6. Various critical material attributes along with their levels for screening by Combined D-optimal design

Independent variables (CMA)		Unit	Levels		
			-1	0	+1
A:	Amount of P90G	mM	10	15	20
B:	Amount of P90H	mM	0	5	10
C:	Surfactant concentration	Mole %	5	10	15
D:	Amount of Drug	Mole %	5	7.5	10

A randomized matrix of twenty eight batches was generated by Design-Expert and presented in **Table 5B-7**. These batches were formulated as per their run order and evaluated for CQA using the methods described earlier. **Table 5B-7** also represents the resulting CQA of these batches.

Table 5B-7. Randomized design matrix for Combined D-optimal response surface design

Batch no.	Run order	Independent Variables				CQA	
		A	B	C	D	Vesicular size (nm)	Drug entrapment (%)
F_1	18	10.0	10.0	15.0	5.0	93.33	72.70
F_2	14	20.0	0.0	15.0	5.0	73.43	67.01
F_3	5	20.0	0.0	5.0	10.0	85.31	58.13
F_4	28	10.0	10.0	5.0	10.0	102.39	57.58
F_5	13	10.0	10.0	5.0	6.5	94.63	63.42
F_6	3	20.0	0.0	5.0	5.0	83.12	60.21
F_7	9	20.0	0.0	15.0	10.0	78.39	63.57
F_8	8	20.0	0.0	10.0	10.0	82.46	65.52
F_9	1	10.0	10.0	8.1	5.0	93.06	73.63
F_{10}	23	10.0	10.0	15.0	10.0	91.83	69.12
F_{11}	17	15.0	5.0	15.0	5.0	69.26	83.41
F_{12}	11	15.0	5.0	15.0	10.0	69.46	78.78
F_{13}	26	20.0	0.0	6.0	7.5	81.10	63.99
F_{14}	24	10.0	10.0	10.7	7.8	92.78	69.99
F_{15}	4	15.0	5.0	10.6	7.2	66.20	81.56
F_{16}	27	15.0	5.0	5.0	5.0	72.19	80.62
F_{17}	7	15.0	5.0	8.0	10.0	71.65	78.67
F_{18}	2	15.0	5.0	5.0	8.5	72.36	76.72

Batch no.	Run order	Independent Variables				CQA	
		A	B	C	D	Vesicular size (nm)	Drug entrapment (%)
F_{19}	15	20.0	0.0	14.2	7.5	73.53	64.67
F_{20}	10	10.0	10.0	8.8	9.5	92.07	68.41
F_{21}	20	12.6	7.4	15.0	7.5	72.02	77.12
F_{22}	21	17.5	2.5	9.7	5.0	74.56	75.79
F_{23}	19	20.0	0.0	10.0	7.7	77.92	69.04
F_{24}	16	10.0	10.0	15.0	10.0	100.79	72.82
F_{25}	12	10.0	10.0	15.0	5.0	89.64	76.85
F_{26}	22	15.0	5.0	15.0	5.0	68.54	86.83
F_{27}	25	10.0	10.0	8.1	5.0	88.94	72.86
F_{28}	6	20.0	0.0	15.0	5.0	72.62	65.96

Based on the experimental data of vesicular size, software suggested quadratic model for mix order and linear model for process order. Analysis of variance (ANOVA) was performed by the software for suggested models. Model terms with a p-value less than or equal to 0.05 (α-level) were considered as significant while terms with higher p-value were considered insignificant. Hierarchy based removal of insignificant model terms was done to simplify the model.

ANOVA and coded coefficients of Full as well as reduced model for vesicular size are presented in **Table 5B-8** and **Table 5B-9**, respectively.

Table 5B-8. Analysis of variance of full as well as reduced quadratic model for vesicular size

Source	Full model					Reduced model (α out - 0.1)*				
	DF	Adj SS	Adj MS	F-Value	P-Value	DF	Adj SS	Adj MS	F-Value	P-Value
Model	8	2902.16	362.77	40.60	< 0.0001	4	2856.07	714.02	76.09	< 0.0001
Linear Mixture	1	1139.04	1139.04	127.49	< 0.0001	1	1139.04	1139.04	121.38	< 0.0001
AB	1	1451.04	1451.04	162.42	< 0.0001	1	1483.15	1483.15	158.05	< 0.0001
AC	1	112.93	112.93	12.64	0.0021	1	157.48	157.48	16.78	0.0004
AD	1	20.63	20.63	2.31	0.1451					
BC	1	10.58	10.58	1.18	0.2902					
BD	1	65.64	65.64	7.35	0.0139	1	50.70	50.70	5.40	0.0665
ABC	1	0.85	0.85	0.095	0.7616					
ABD	1	29.47	29.47	3.30	0.0852					
Residual	19	169.75	8.93			23	215.84	9.38		
Lack-of-Fit	14	113.68	8.12	0.72	0.7101	18	159.77	8.88	0.79	0.6779
Pure Error	5	56.07	11.21			5	56.07	11.21		
Total	27	3071.90				27	3071.90			

* Shaded rows represent insignificant model terms removed during model reduction.

ANOVA table for vesicular size showed insignificant quadratic effect among selected CMA. However, the linear mixture and interactive effects between P90G and P90H as well as P90G and Surfactant were found to affect vesicular size significantly. An insignificant lack-of fit showed the adequacy of the model in explaining the variation in the responses.

Table 5B-9. Coded coefficients of full as well as reduced quadratic model for vesicle size

Term	Full Model			Reduced model (α out - 0.1)*		
	Coef	SE Coef	VIF	Coef	SE Coef	VIF
A-90G	79.35	1.00	1.27	79.42	1.02	1.26
B-90H	94.04	0.95	1.25	93.85	0.96	1.22
AB	-66.26	5.20	1.54	-65.42	5.20	1.47
AC	-4.30	1.21	1.27	-4.56	1.11	1.02
AD	1.86	1.22	1.32			
BC	-1.29	1.19	1.30			
BD	2.91	1.07	1.18	2.36	1.02	1.01
ABC	1.90	6.19	1.54			
ABD	-11.16	6.14	1.51			

* Shaded rows represent insignificant model terms removed during model reduction

Coefficients table for vesicular size showed VIF values near to 1 indicating that the predictors are not correlated and regression coefficients are well estimated. Regression equations for full and reduced models in uncoded units are presented as **Eq. 5B-2** and **Eq. 5B-3**, respectively. The positive and negative sign before each coefficients indicates a direct or inverse relationship of that model term with vesicle size.

Full model

$$R1 = 82.38A + 166.62B - 146.37AB - 0.86AC + 0.74AD - 0.42BC + 10.51BD + 1.52ABC - 17.85ABD$$

Eq. 5B-2

Reduced model

$$R1 = 88.53A + 215.82B - 261.69AB - 0.91AC + 0.91BC + 1.89BD$$ **Eq. 5B-3**

Based on the experimental data of drug entrapment, software suggested quadratic model for both mix order as well as process order. ANOVA and coded coefficients of Full as well as reduced quadratic model for drug entrapment are presented in **Table 5B-10** and **Table 5B-11**, respectively.

Table 5B-10. Analysis of variance of full as well as reduced quadratic model for drug entrapment

Source	Full model					Reduced model (α out - 0.1)*				
	DF	Adj SS	Adj MS	F-Value	P-Value	DF	Adj SS	Adj MS	F-Value	P-Value
Model	17	1577.46	92.79	21.39	< 0.0001	10	1551.70	155.17	38.15	< 0.0001
Linear Mixture	1	121.69	121.69	28.05	0.0003	1	121.69	121.69	29.92	< 0.0001
AB	1	92.33	92.33	21.28	0.0010	1	149.88	149.88	36.85	< 0.0001
AC	1	36.07	36.07	8.31	0.0163	1	37.41	37.41	9.20	0.0075
AD	1	6.60	6.60	1.52	0.2455	1	13.05	13.05	3.21	0.0911
BC	1	124.91	124.91	28.79	0.0003	1	161.16	161.16	39.61	< 0.0001
BD	1	67.74	67.74	15.61	0.0027	1	74.83	74.83	18.40	0.0005
ABC	1	16.63	16.63	3.83	0.0787	1	18.43	18.43	4.53	0.0482
ABD	1	0.04	0.04	0.01	0.9272					
ACD	1	0.55	0.55	0.13	0.7295					
BCD	1	9.14	9.14	2.11	0.1772					
AC^2	1	22.11	22.11	5.10	0.0476	1	39.20	39.20	9.64	0.0064
AD^2	1	3.22	3.22	0.74	0.4092					
BC^2	1	35.51	35.51	8.18	0.0169	1	42.56	42.56	10.46	0.0049
BD^2	1	6.27	6.27	1.44	0.2572					
ABCD	1	5.58	5.58	1.29	0.2831					
ABC^2	1	12.92	12.92	2.98	0.1151	1	19.98	19.98	4.91	0.0406
ABD^2	1	0.66	0.66	0.15	0.7050					
Residual	10	43.38	4.34			17	69.14	4.07		
Lack of Fit	5	21.22	4.24	0.96	0.5185	12	46.98	3.91	0.88	0.6050
Pure Error	5	22.17	4.43			5	22.17	4.43		
Cor Total	27	1620.84				27	1620.84			

* Shaded rows represent insignificant model terms removed during model reduction

ANOVA table for drug entrapment showed significant interaction, quadratic and linear mixture effects among selected CMA. Significant quadratic terms indicated that the relationship between these CMA and drug entrapment follow a curved line. An insignificant lack-of fit showed the adequacy of the model in explaining the variation in the responses.

Table 5B-11. Coded coefficients of full as well as reduced quadratic model for drug entrapment

Term	Full Model			Reduced model (α out - 0.1)*		
	Coef	SE Coef	VIF	Coef	SE Coef	VIF
A-90G	68.04	1.52	6.09	67.68	1.37	5.26
B-90H	70.26	1.74	8.66	72.09	1.15	4.04
AB	41.42	8.98	9.46	41.60	6.85	5.88
AC	2.46	0.85	1.30	2.48	0.82	1.27
AD	-1.14	0.92	1.54	-1.38	0.77	1.14
BC	4.95	0.92	1.62	5.36	0.85	1.47
BD	-3.20	0.81	1.38	-2.97	0.69	1.08
ABC	-9.18	4.69	1.82	-9.03	4.24	1.59
ABD	0.44	4.72	1.84			

Term	Full Model			Reduced model (α out - 0.1)*		
	Coef	SE Coef	VIF	Coef	SE Coef	VIF
ACD	-0.35	0.97	1.32			
BCD	1.40	0.96	1.40			
AC^2	-4.59	2.03	6.90	-5.35	1.72	5.29
AD^2	-1.42	1.65	4.90			
BC^2	-4.81	1.68	5.12	-4.97	1.54	4.57
BD^2	2.34	1.95	7.62			
ABCD	-5.94	5.24	1.71			
ABC^2	16.43	9.52	7.32	18.71	8.44	6.14
ABD^2	3.63	9.33	7.00			

* Shaded rows represent insignificant model terms removed during model reduction

Coefficients table for drug entrapment showed VIF values near to 1 for 2-way as well as 3-way interaction terms while it was <10 for quadratic terms indicating that the predictors are not correlated and regression coefficients are well estimated. Regression equations for full and reduced models in uncoded units are presented as **Eq. 5B-4** and **Eq. 5B-5**, respectively. The positive and negative sign before each coefficients indicates a direct or inverse relationship of that model term with drug entrapment.

Full model

$$R2 = 33.32A - 115.52B + 484.97AB + 4.37AC + 3.23AD + 26.47BC - 11.67BD - 45.67ABC - 15.16ABD - 0.03ACD + 1.20BCD - 0.18AC^2 - 0.23AD^2 - 1.52BC^2 - 0.19BD^2 - 1.90ABCD + 2.63ABC^2 + 2.33ABD^2$$

Eq. 5B-4

Reduced model

$$R2 = 45.45A - 213.70B + 538.09AB + 4.78AC - 0.55AD + 38.88BC - 1.83BD - 67.11ABC - 0.21AC^2 - 1.68BC^2 + 2.99ABC^2$$

Eq. 5B-5

Model summary for both CQA is presented in **Table 5B-12**. A low SD value and high R^2 value indicated a better prediction of responses by the model. Predicted R^2 was found to be in good agreement with other R^2 further supporting the prediction potential of the model.

Table 5B-12. Summary of full as well as reduced quadratic-linear model for both CQA

Responses	Full model				Reduced model (α out - 0.1)			
	SD	R-sq	R-sq (adj)	R-sq (pred)	SD	R-sq	R-sq (adj)	R-sq (pred)
Vesicle size (nm)	2.99	94.47	92.15	88.21	3.06	92.97	91.75	89.56
Drug entrapment (%)	2.08	97.32	92.77	75.57	2.02	95.73	93.22	89.93

Four different residual plots viz., normal plot of residual, residual versus ascending predicted response values, residual versus experimental run order and predicted versus actual were generated for both CQA and presented in **Fig. 5B-4** and **Fig. 5B-5**.

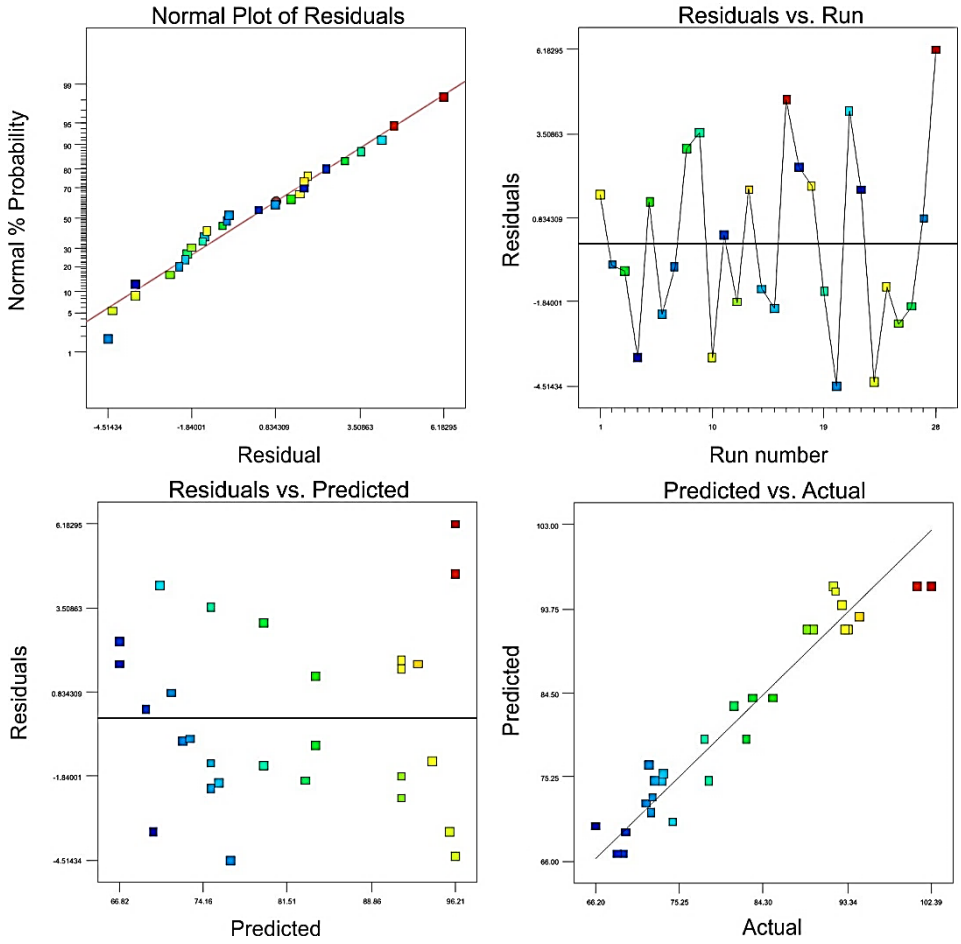

Fig. 5B-4. Residual plots for vesicle size

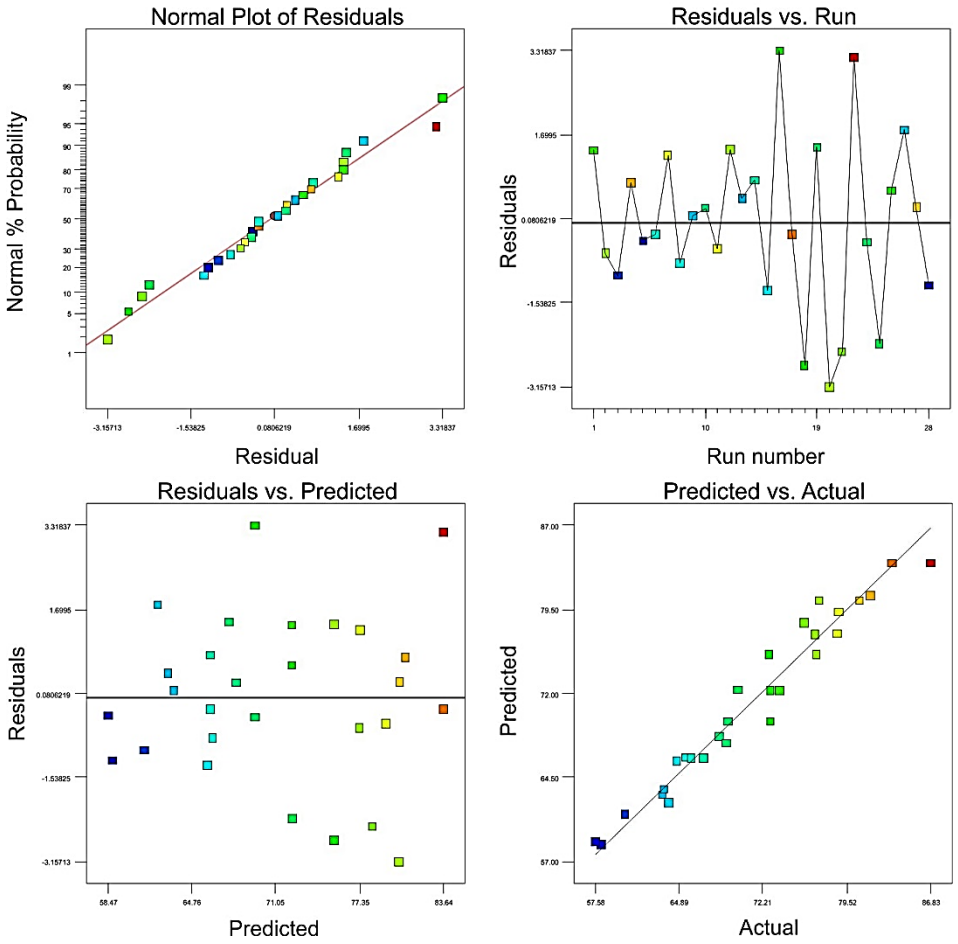

Fig. 5B-5. Residual plots for drug entrapment

In normal plot, residuals were appeared to follow a straight line indicating that the data was normally distributed. Random scattering without any megaphone pattern in residual versus predicted plot validated the assumption of constant variance. Similarly, random scattering without any pattern in residual versus run plot validated the absence of lurking variables. In predicted versus actual plot, data points were evenly split by the 45-degree line indicating easy prediction of values by the model [6].

The main effect plots for both CQA are presented in **Fig. 5B-6**. These graphs provided a better depiction of how the individual CMA influence respective CQA and found in-line with the ANOVA results.

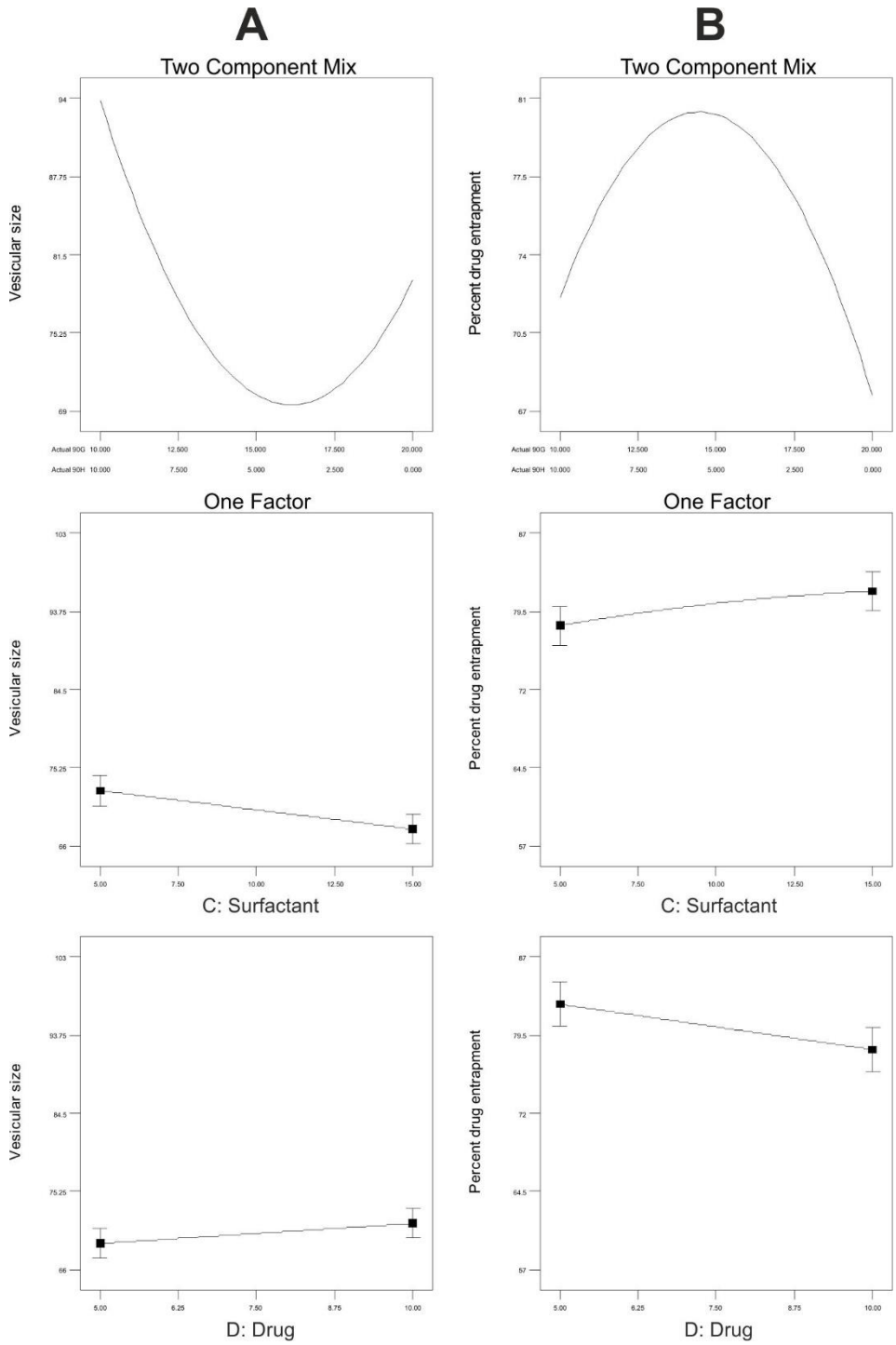

Fig. 5B-6. Main effect plots of reduced model for A. vesicular size and B. drug entrapment

Contour and response surface plots are presented in **Fig. 5B-7** and **Fig. 5B-8**, respectively. These graphs were used to depict how the CQA is related to respective CMA while keeping other CMA at constant levels.

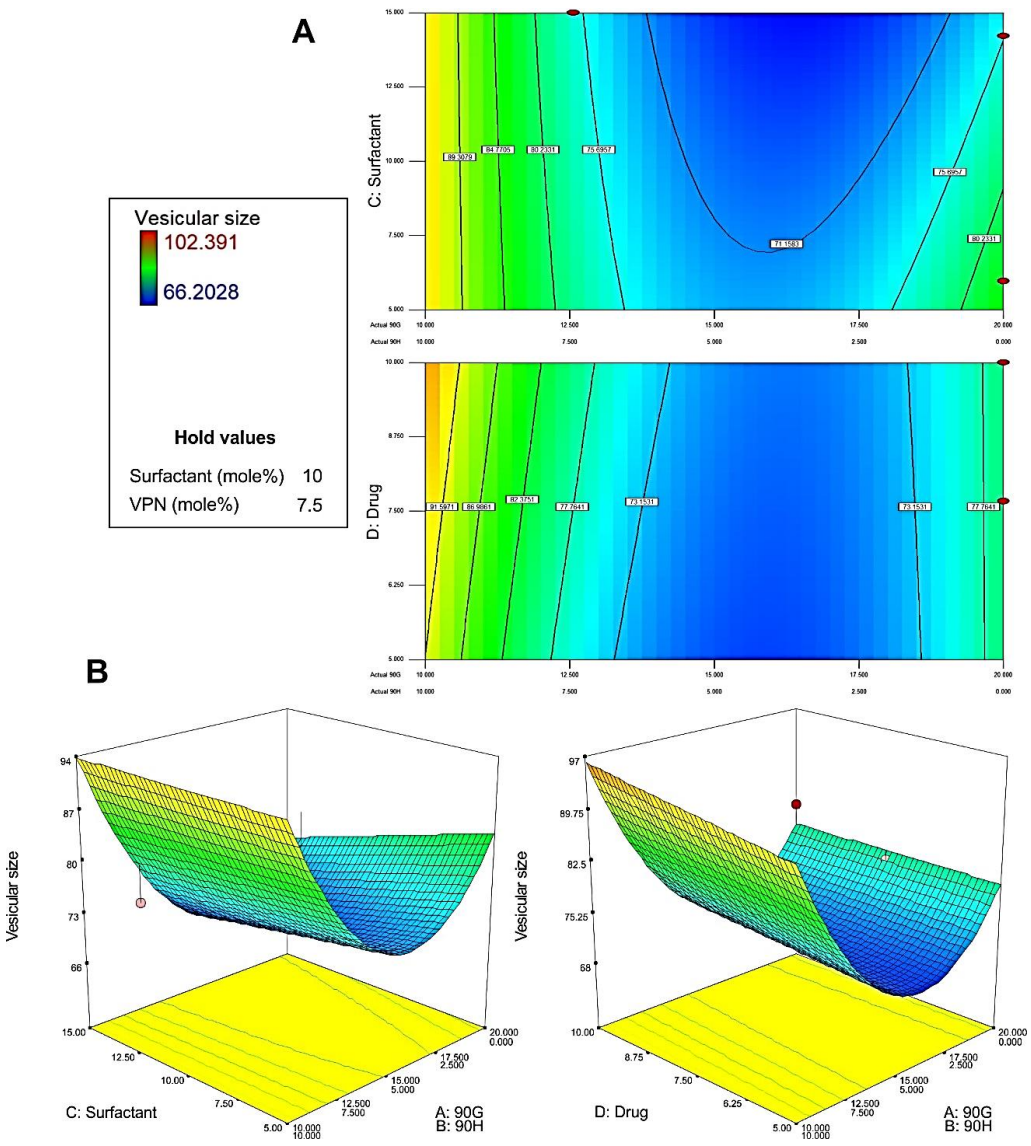

Fig. 5B-7. Contour and response surface plots of reduced model for vesicular size

Numerical optimization was performed by the software for defined optimization criteria as presented in **Table 5B-13**. The software was programmed to provide the optimization solution with minimum vesicle size and maximum drug entrapment while keeping all the CMA within experimental range.

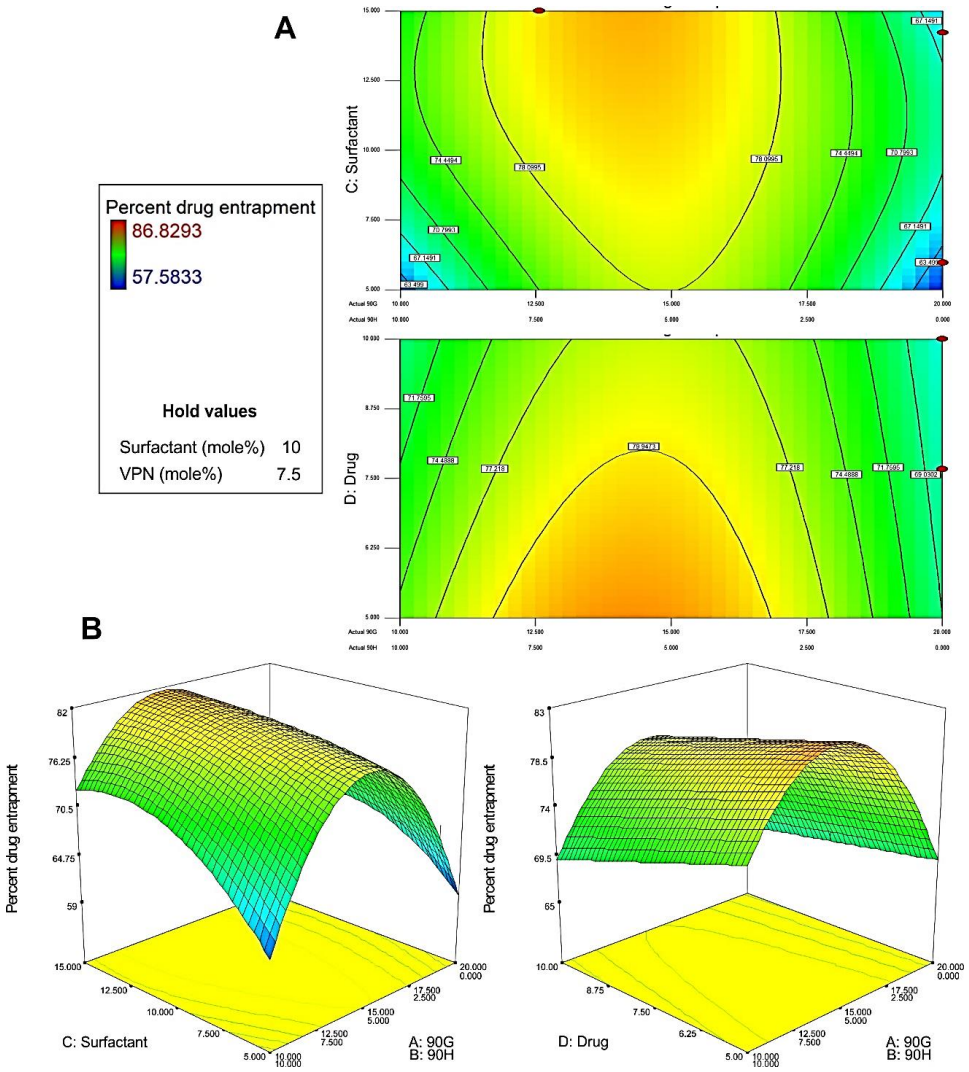

Fig. 5B-8. Contour and response surface plots of reduced model for drug entrapment

Table 5B-13. Criteria for optimization of NPT UDL

Constraints name	Goal	Lower	Upper	Weight	Importance
P90G	in range	10	20	1	3
P90H	in range	0	10	1	3
Surfactant	in range	5	15	1	3
Drug	in range	5	10	1	3
Vesicular size	minimize	66.20	102.39	1	3
Percent drug entrapment	maximize	57.58	86.83	1	3

Overlaid contour plots for both CQA were generated (**Fig. 5B-9**) to observe the design space (yellow area in graph) using the defined criteria shown in above table.

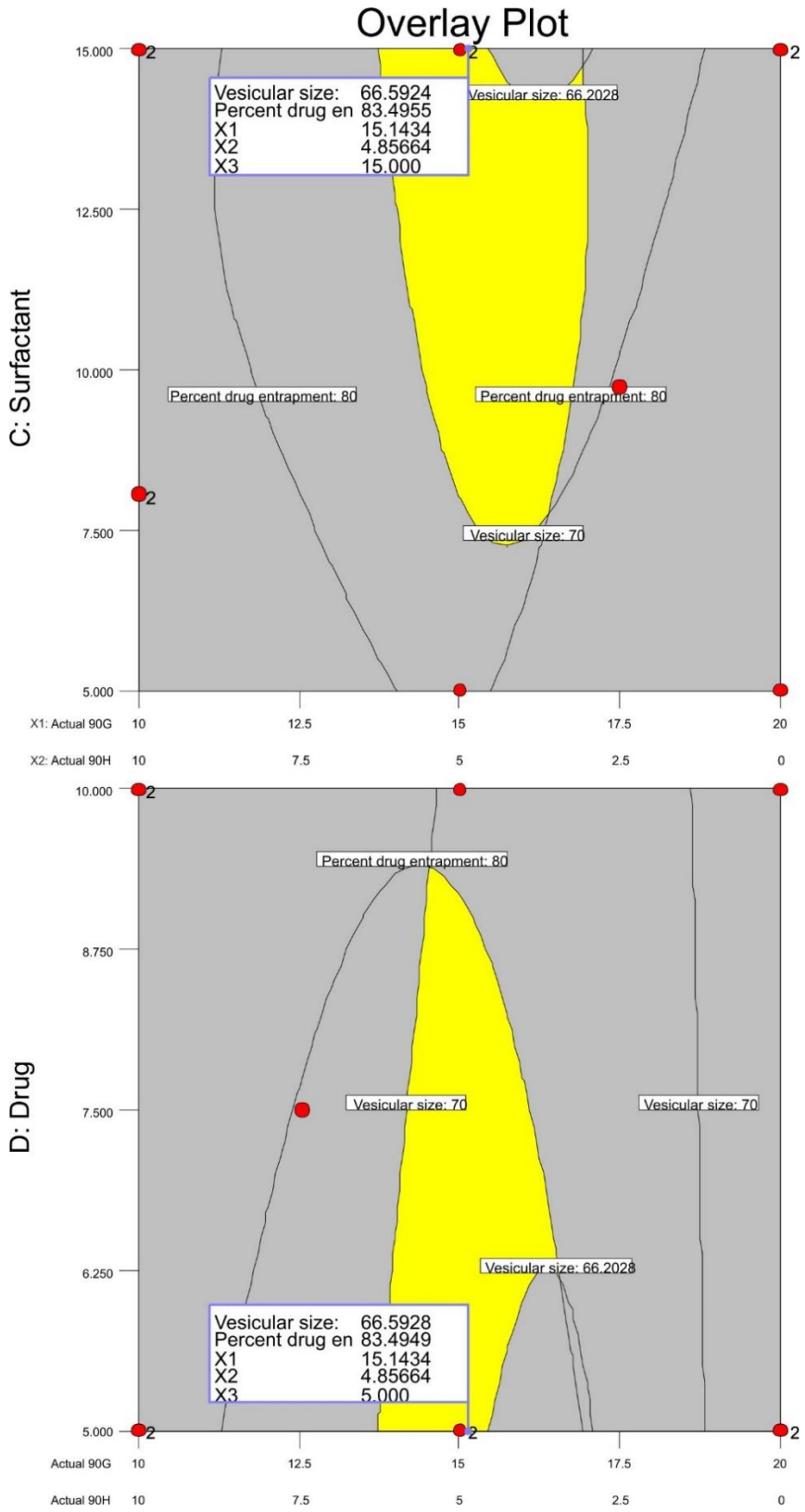

Fig. 5B-9. Overlaid contour plots of reduced quadratic model showing design space

Further, the optimization solution ramp and desirability plot are presented in **Fig. 5B-10** and **Fig. 5B-11**.

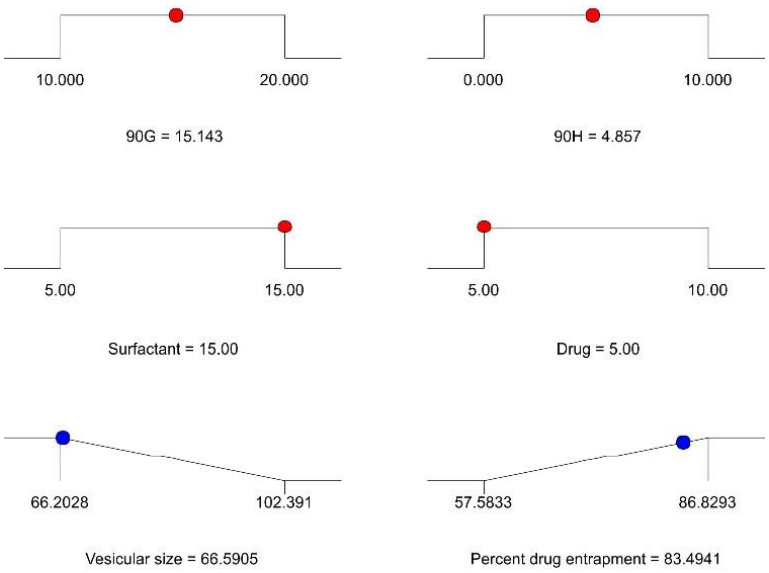

Fig. 5B-10. Optimization solution ramp for defined desirability criteria

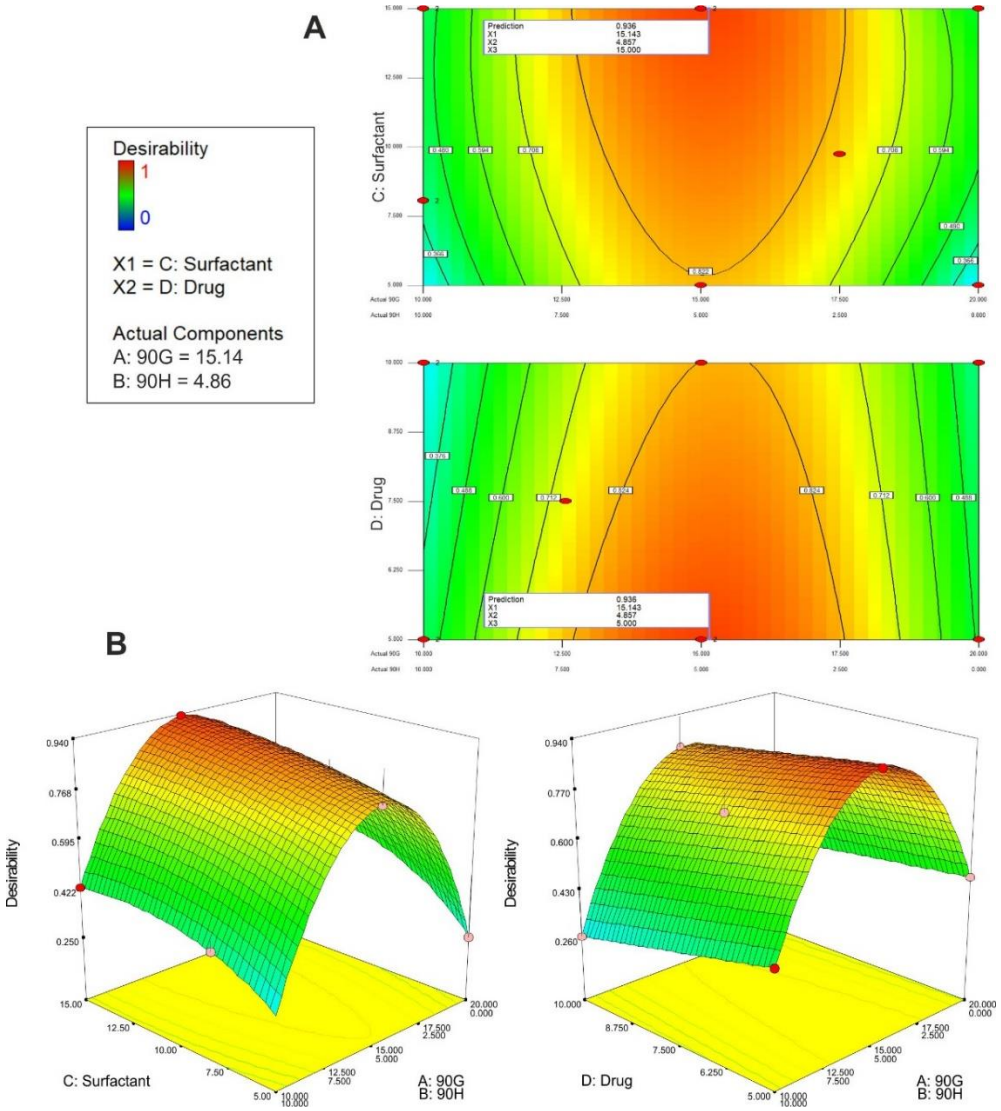

Fig. 5B-11. A) Contour and B) Response surface plot of composite desirability for both CQA

The optimization solution ramp and desirability plot showed a composite desirability of 0.936 for the solution provided by the software. The setting of this optimization solution is also presented in **Table 5B-14** along with the 95% confidence as well as 95% prediction intervals. Three batches with optimized levels were prepared for verification trials and the values of both CQA are presented in **Table 5B-15**.

Table 5B-14. Optimization solution

Multiple Response Prediction

Variable	Setting
P90G (mM)	15.14
P90H (mM)	4.86
Surfactant (mole%)	15
Drug (mole%)	5

Responses	Prediction	SE Mean	95% Confidence interval		95% Prediction interval	
			Lower	Upper	Lower	Upper
Vesicular size (nm)	66.59	1.26	63.99	69.19	59.74	73.44
Drug entrapment (%)	83.49	1.11	81.15	85.84	78.63	88.35

Table 5B-15. Results of verification trials

Responses	95% Prediction interval		Results			
	Lower	Upper	Batch-1	Batch-2	Batch-3	Average
Vesicular size (nm)	59.74	73.44	70.06	64.57	66.41	67.01
Drug entrapment (%)	78.63	88.35	81.85	83.68	82.42	82.65

The average values of both CQA were found to fall within 95% confidence interval and thus indicated the validity of the model.

5B.3.2 *In vitro* characterization of optimized NPT UDL

5B.3.2.1 Shape and surface morphology

Transmission electron microscopy of optimized NPT UDL was performed and the image is represented as **Fig. 5B-12**. The image showed spherical shape with smooth surface of liposomes. The size of liposomes seen in the image was found in-line with the results of vesicular size data obtained from Malvern zetasizer (**Fig. 5B-13**).

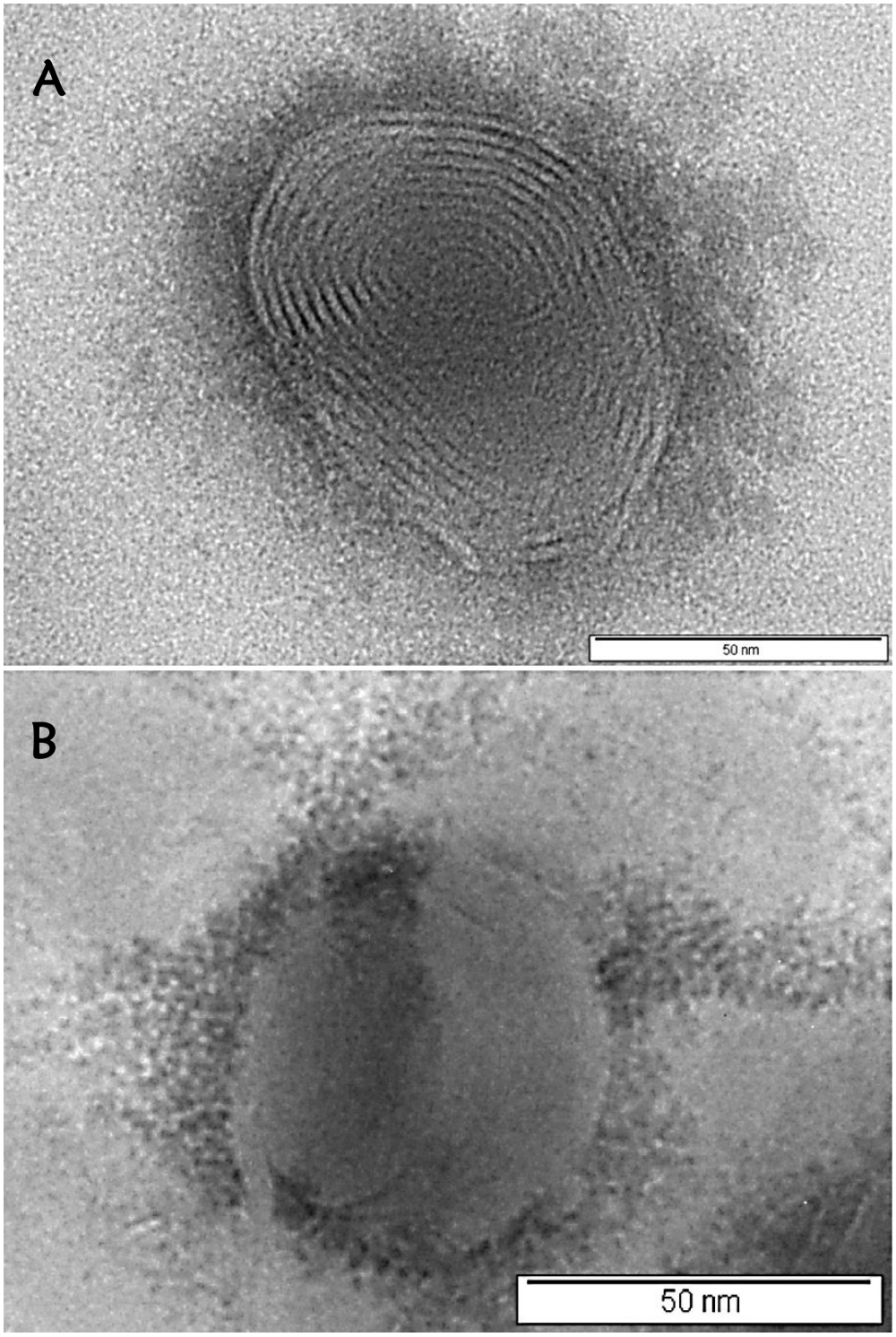

Fig. 5B-12. Transmission electron microscopic images showing (A) multi-lamellae and (B) surface of optimized NPT UDL

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	271.3	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	6

Results

			Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm):	68.5	**Peak 1:**	70.4	100.0	124.5
PdI:	0.189	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.955	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

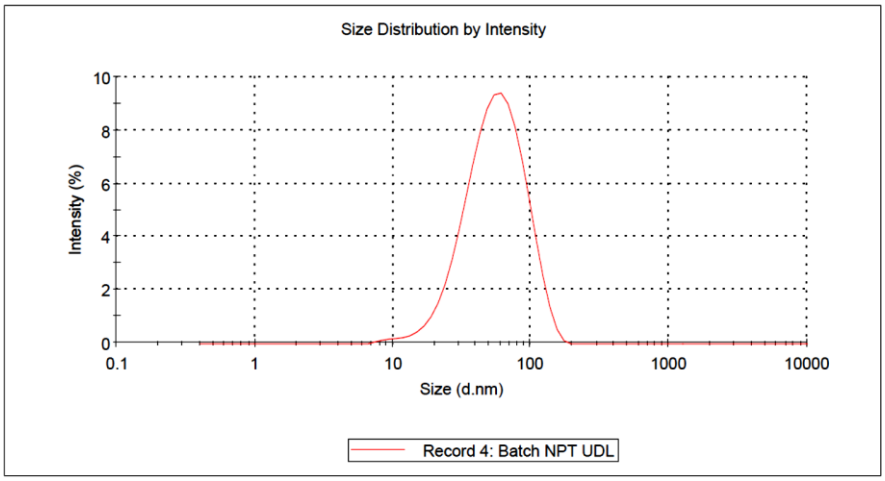

Fig. 5B-13. Size distribution graph of optimized NPT UDL

System

Temperature (°C):	25.0	**Zeta Runs:**	13
Count Rate (kcps):	74.9	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	10

Results

			Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	-29.5	**Peak 1:**	-29.5	100.0	6.39
Zeta Deviation (mV):	6.39	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0640	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

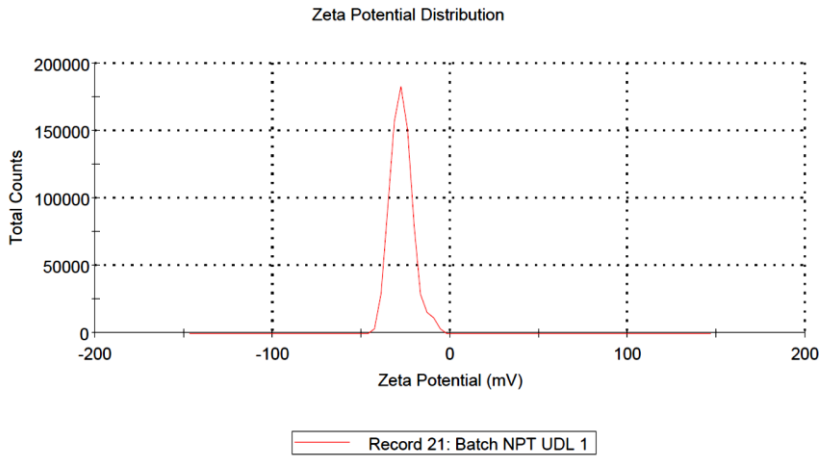

Fig. 5B-14. Zeta potential graph of optimized NPT UDL

5B.3.2.2 Zeta potential

The zeta potential graph of optimized NPT UDL (**Fig. 5B-14**) showed a net negative charge on liposome surface with a zeta potential value of -29.5 mV. The charge was found sufficient enough to keep the particles dispersed via repulsive forces [7, 8].

5B.3.2.3 In-vitro drug release study

In vitro drug release from NPT UDL was evaluated and the cumulative percent drug release at different time points are summarized in **Table 5B-16** as well as illustrated in **Fig. 5B-15**. In order to ensure that the presence of NPT in release media directly reflects its release from nanocarriers, the permeation of released NPT across dialysis membrane should not be rate limiting. Hence, data for NPT solution was also generated which showed >90% drug release within 2 hours indicating non-barrier nature of dialysis membrane for dissolved NPT. Release data of NPT UDL showed >50 % NPT release in first 8 hours and > 80 % release in 24 hours indicating the control release behavior of UDL.

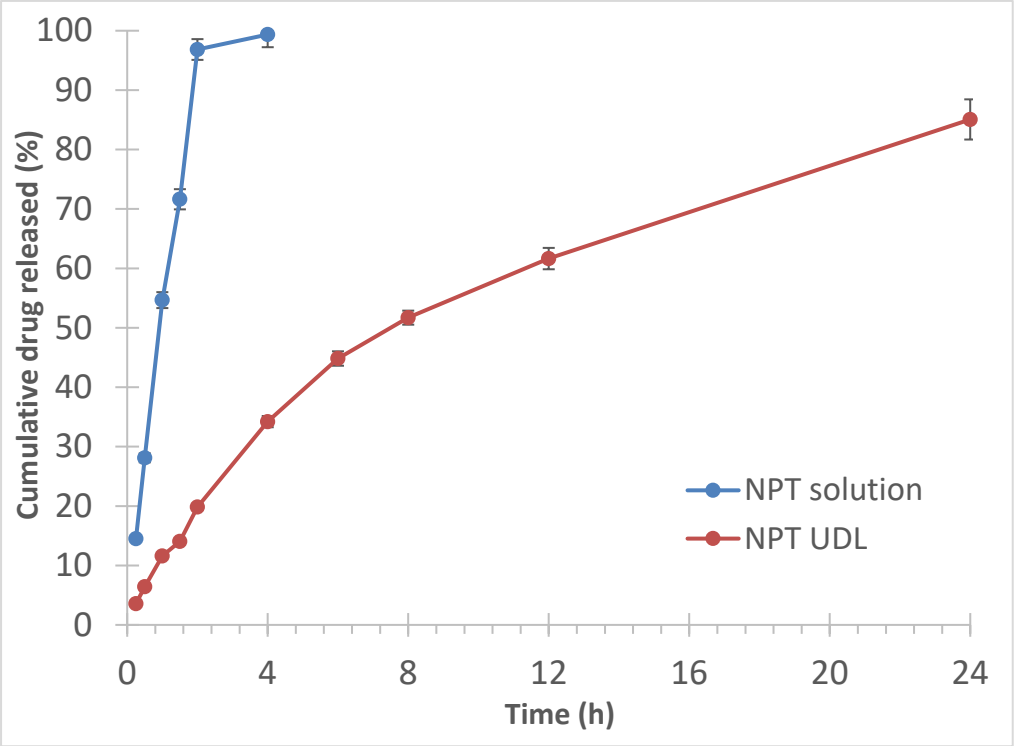

Fig. 5B-15. Cumulative percent of NPT released *in vitro* versus time curve for UDL

Table 5B-16. *In vitro* release profile of NPT from its solution and UDL

Time (h)	Cumulative percent drug released	
	NPT Solution*	NPT UDL*
0.25	14.52 ± 0.48	3.58 ± 0.12
0.5	28.12 ± 0.96	6.42 ± 0.23
1	54.66 ± 1.05	11.61 ± 0.39
1.5	71.63 ± 2.46	14.06 ± 0.54
2	96.84 ± 3.85	19.84 ± 0.24
4	99.36 ± 1.70	34.21 ± 0.43
6	-	44.83 ± 1.61
8	-	51.71 ± 1.55
12	-	61.65 ± 1.46
24	-	85.06 ± 1.05

* Result represented as mean ± SD

The result of various mathematical models, applied to understand the NPT release kinetics from UDL, are presented in Table 5B-17.

Table 5B-17. Various mathematical models and their correlation coefficient values

Mathematical models	Graph description (Y-axis versus X-axis)	NPT UDL	
		R^2	n
Zero order	Cumulative amount/percent of drug released *versus* time	0.947	-
First order	Log cumulative percent drug remaining *versus* time	-0.998	-
Higuchi	Cumulative percent drug released *versus* square root of time	0.996	-
Hixon Crowell	Cube root of percent drug remaining *versus* time	-0.988	-
Korsmeyer Peppas	Log cumulative percent drug released *versus* log time	0.993	0.75

The R^2 values for Higuchi as well as first order model was found higher suggesting a diffusion controlled system where release rate was dependent on remaining drug concentration within the carrier.

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