Chapter 5

FORMULATION

DEVELOPMENT

5.1 Introduction

The aim of pharmaceutical development is to design a quality product and its manufacturing process to consistently achieve the intended performance of the product. Quality of pharmaceutical products is the most important requirement of all the regulatory bodies. Customer satisfaction with regard to process, product and service, solely depends on quality of the product. Quality of a product is interrelated with cost, time and productivity. Hence, it is essential to build quality in the product by accurate planning, so as to avoid forth coming failures. Meagre analysis of final product will not be sufficient, however, quality should be imbibed in it from initial stage. Quality by Design (QbD) have been utilized to design the product and its process. QbD means designing and developing acceptable formulation along with its manufacturing process to ensure a product with predefined quality attributes [1].

Design of experiments (DOE) is one of the tool used for building quality in the formulation. DOE was firstly introduced by Sir Ronald Fisher in his book "The Design of Experiments" back in 1935 [2]. DOE is a constructive and organized method to analyze the relationship between the factors that affect the outcomes of a process. It has been recommended that DOE can provide accurate predictions with fewer experiments. Optimization of a pharmaceutical process instigate an objective to identify and assess independent variables which influence formulation response, determination and establishing their best fit values. Development and optimization of a pharmaceutical product involves setting various process and formulation variables which directly affects the safety and efficacy. Various variables are screened stepwise and their statistical significance is determined by using polynomial non-linear regression analysis. Statistically fit model would be used to forecast the relationship between the selected variables and their levels [3, 4]. Optimization can be done by changing one-variable-at-a-time, however, it is a complex method where effects of other variables on the outcome of the experiment cannot be assessed. Another method is to precisely evaluate the influence of pre-identified independent variables on the dependent variables by simultaneously varying all the key factors in an organized manner. This approach is known as response surface methodology (RSM). It is a statistical technique, which can be applied to understand the relationship between several independent and related dependent variables. RSM provides significant amount of information. Moreover, it is an economical approach as fewer number of experiments are required to observe the effect of independent variables over selected response variables. Various RSM are used for optimization such as Full factorial, Box-Behnken, Central composite and Doehlert [5]. In each methodology number of experiments is dependent on the number of independent variables.

In the present investigation, several independent variables were studied in preliminary trials, which might affect the size and % entrapment efficiency (%EE) of spherulites. Three critical independent parameters were carefully chosen; Phospholipid concentration (%w/w), Hydration time (Hours (hrs)) and Probe-Cylinder distance (mm). Gemcitabine hydrochloride (GCH) loaded spherulites were optimized by applying 3^3 full factorial design. Whereas, Vinorelbine tartrate (VLB) loaded spherulites were optimized using Box-Behnken design. Each independent factor was studied at three levels i.e. low (-1), intermediate (0) and high (+1). The polynomial equation was obtained by analyzing the response variables of formulation (size and %EE).

5.2 Materials and Equipment

5.2.1 Materials

Gemcitabine HCl (GCH) was obtained as a gift sample from Sun Pharmaceutical Industries Ltd., Vadodara, India. Vinorelbine tartrate (VLB) was obtained as a gift sample from Cipla Ltd. Mumbai, India. Cholesterol (Chol), Mannitol and Potassium oleate were purchased from Sigma Aldrich (St. Louis, MO, USA). Soyabean Phosphatidylcholine (SPC) 95% (PhospholiponVR 90 G) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG 2000) were obtained as gift sample from Lipoid GmbH (Ludwigshafen, Germany). Methanol and Chloroform (A. R. grade) were purchased from S.D. Fine-chemicals limited (Vadodara, India). Distilled water was prepared using in-house distillation assembly. 0.22µ membrane filter was purchased from Pall Life Sciences (Mumbai, India). All other reagents were purchased from S.D. Fine-chemicals limited, Baroda, India and were of analytical reagent grade.

5.2.2 Equipments

- Analytical Weighing Balance (ATX 224, Shimadzu, Japan)
- Vortex Mixer (Spinix-Vortex Shaker, Tarsons, India)
- Ultrasonic Bath Sonicator (Ultrasonics Selec, Vetra, Italy)
- Rotary evaporator (IKA RV10, Karnataka, India)
- Particle Size Analyzer 3000 HS (Zeta Sizer Nano Series, Malvern Instruments, UK)
- UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan)
- Cooling Centrifuge (Remi Equipment, Mumbai, India)
- Nikon H600L Microscope (Nikon, Japan)
- Transmission Electron Microscope Tecnai 20 (Philips, Holland)

5.3 Preparation and Optimization of GCH loaded Spherulites

5.3.1 Preparation of spherulites formulation and optimization by Design of Experiment®

GCH loaded spherulites were prepared and optimized by applying 3^3 Full Factorial design. Spherulites were composed of SPC, Chol and Potassium oleate. Upon preliminary investigation (Chapter 4: Preformulation studies) independent variables were chosen viz. Phospholipid concentration (%w/w), Hydration time (hrs) and Probe-Cylinder distance (mm). While, spherulites size and %EE were response variables. Independent variables and their levels studied in 3^3 Full Factorial design are shown in Table 5.1. Figure 5.1 shows the developmental process of GCH loaded Spherulites.

Table 5.1: Independent variables and their levels studied in 3^3 Full Factorial design.

Independent variables	Levels		
	Low (-1)	Intermediate (0)	High (+1)
Phospholipid concentration (%w/w)	34	38	42
Hydration time (hrs)	12	18	24
Probe-Cylinder distance (mm)	0.10	0.55	1.0

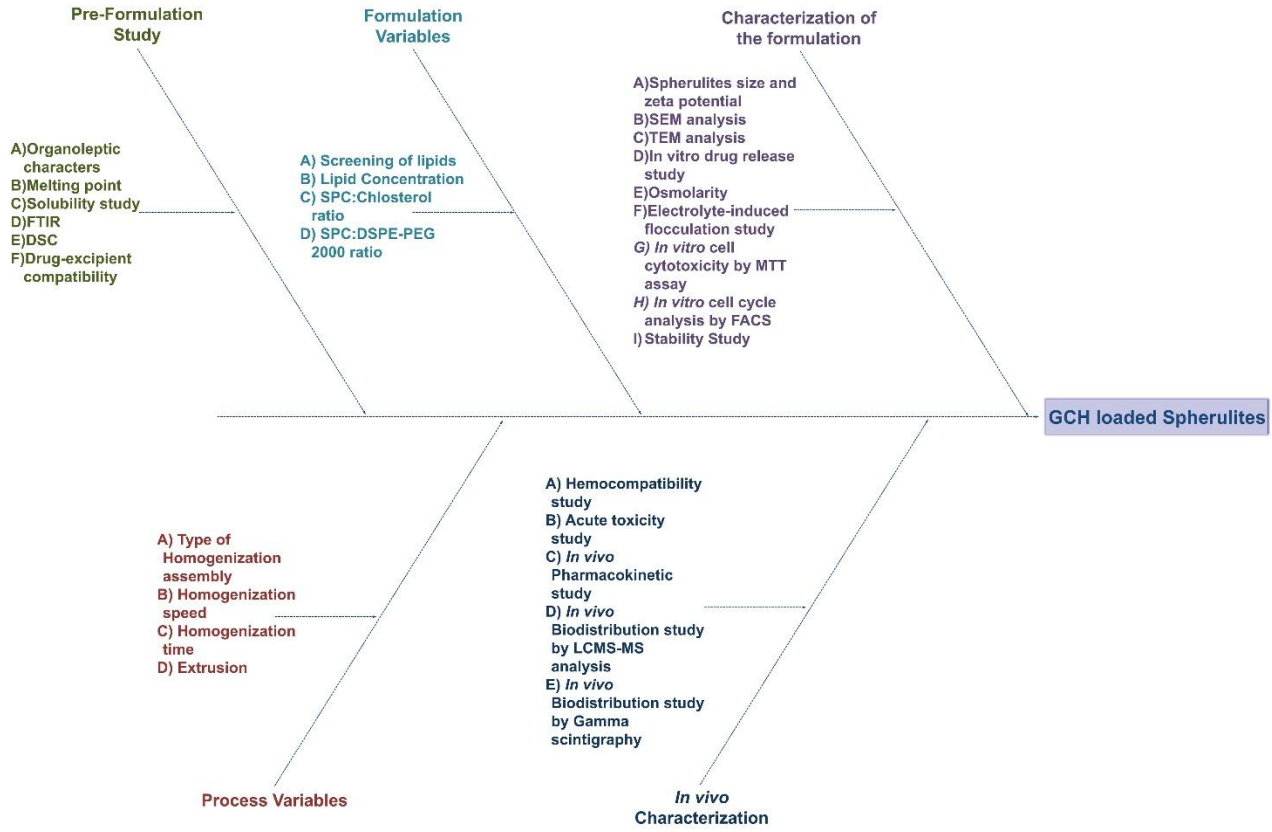

Figure 5.1: Ishikawa diagram showing the developmental process of GCH loaded Spherulites.

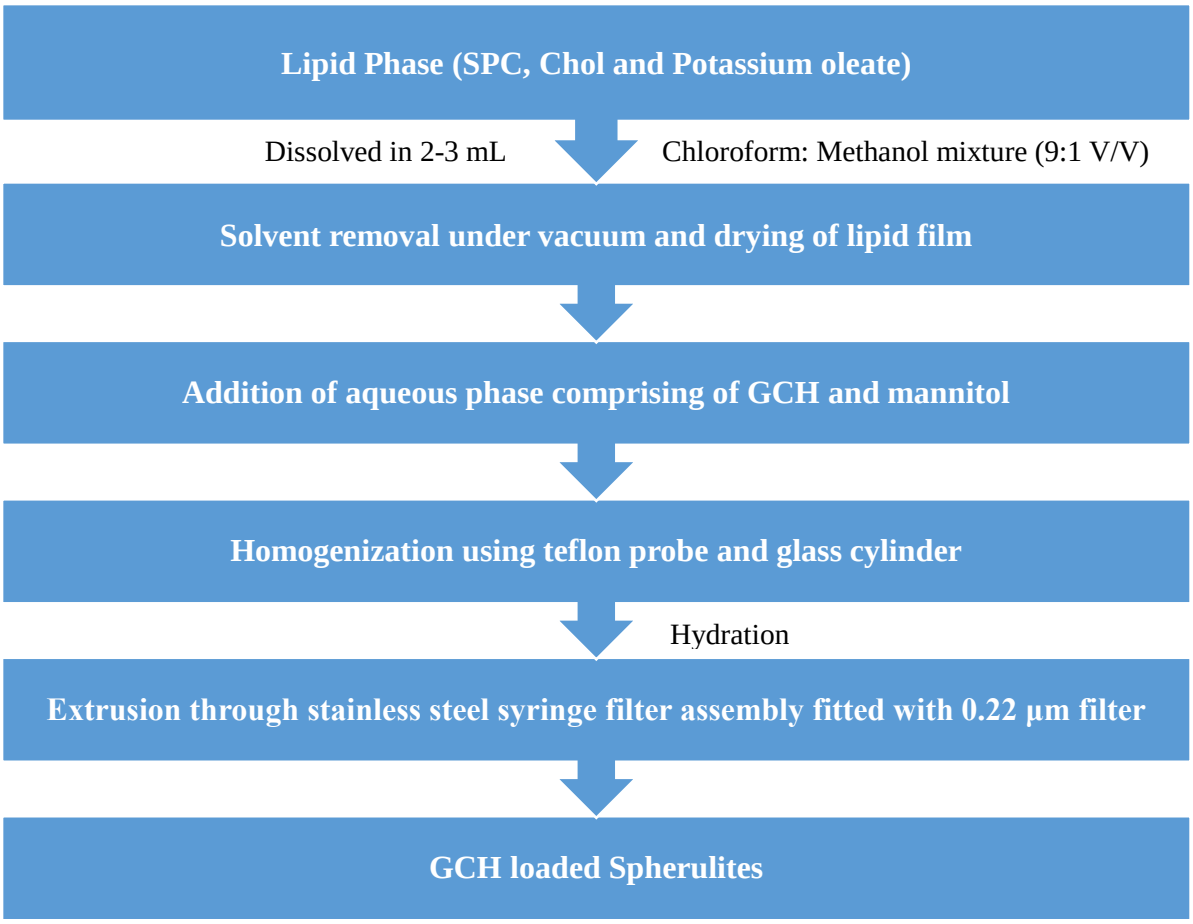

Figure 5.2: Flow chart depicting the preparation of GCH loaded Spherulites.

The non-PEGylated spherulites were composed of SPC/Chol. Lipid phase comprising of SPC: Chol (1:1 molar) along with potassium oleate, were dissolved in 2-3 ml of chloroform: methanol mixture (9:1 V/V). The organic solvent was removed under vacuum and the resulting film was dried using a rotary evaporator (IKA RV10, Bengaluru, India) at 60 ºC for 15 minutes. 20 mL aqueous phase comprising mannitol and 10 mg of GCH was used to hydrate the film. The resulting dispersion was kept for hydration. Hydrated dispersion was homogenized (Eurostar power control-visc, IKA, Bengaluru, India) using a custom designed assembly of teflon probe and glass cylinder for 1 hour at shear rate of 65 min^{-1}. The sheared dispersion was subjected for extrusion 5 times through stainless steel syringe filter assembly fitted with 0.22 µm filter [6, 7]. Preliminary studies (Chapter 4. Preformulation studies and preliminary optimization) reveled that size of spherulites before extrusion was very large(more than 800nm) therefore, it was necessary to downsize them for developing an acceptable formulation. Figure 5.2 shows the flowchart of preparation procedure of GCH loaded spherulites.

5.3.1.1 Determination of spherulites size

Briefly, 50 µL of dispersion was diluted with 2 ml of distilled water and taken in disposable

polystyrene cuvette. Mean hydrodynamic diameter was measured in triplicate by dynamic light scattering using Malvern size analyzer (Malvern Nanoseries-ZS, Malvern Instruments, United Kingdom).

5.3.1.2 Determination of % Entrapment efficiency (% EE)

Spherulites loaded with GCH were taken in centrifuge tube and centrifuged at 20000 RPM at 4 ºC for 30 minutes (REMI Laboratory Instruments, Mumbai, India). Spherulites pellet settled at the bottom of tube was air dried and weighed for accounting the total weight of solid content. 2% Triton X100 solution was used to lyse the pellet and diluted suitably. Drug amount was estimated using UV spectrophotometer (Shimadzu 1800, Kyoto, Japan) at 266 nm for GCH. Supernatant was also diluted suitably and the amount of free drug was estimated to establish the mass balance.

Calculation of the % Entrapment Efficiency (EE) was performed using following formula:

$$\% EE = \frac{Estimated\ Entrapped\ drug\ in\ Spherulites}{Total\ drug\ added\ during\ formulation} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

3^3 Full Factorial design batches with their measured responses are shown in Table 5.2.

Table 5.2: 3^3 Full Factorial design batches with their measured responses (N=3; Mean±SD).

	Factor A	**Factor B**	**Factor C**	**Response 1**	**Response 2**
Batch	**Phospholipid conc. (% w/w)**	**Hydration time (Hrs)**	**Probe-cylinder distance (mm)**	**%EE (%)**	**Spherulites size (nm)**
1	34	24	0.1	66.40±1.2	245.6±2.1
2	38	24	1	62.41±1.1	228.6±1.9
3	38	18	1	59.28±1.3	224.2±1.8
4	34	12	0.55	78.91±1.1	200.9±1.6
5	42	12	0.1	72.14±1.6	243.2±2.4
6	42	12	0.55	71.97±1.5	210.2±2.7
7	38	12	0.1	68.42±1.4	235.2±1.5
8	42	18	1	69.63±1.1	231.4±1.2
9	34	24	0.55	76.82±1.3	208.4±1.3
10	34	18	0.55	78.36±1.5	202.1±1.1
11	34	18	0.1	71.64±1.9	250.2±2.7
12	38	18	0.1	62.10±2.4	238.9±2.4

13	38	18	0.55	74.24±1.9	200.2±1.8
14	42	18	0.55	76.41±1.4	212.9±1.6
15	42	24	1	66.68±1.8	227.1±2.4
16	38	24	0.55	77.62±1.5	207.6±2.7
17	34	12	1	51.21±1.1	222.5±2.3
18	42	24	0.1	73.74±1.6	243.0±1.5
19	38	12	1	63.33±1.2	234.3±2.9
20	38	12	0.55	73.12±1.3	204.6±1.2
21	42	24	0.55	72.56±1.5	205.3±1.9
22	38	24	0.1	68.25±2.4	252.3±1.8
23	34	18	1	71.29±1.4	235.5±1.6
24	34	12	0.1	72.19±1.6	248.6±2.3
25	42	12	1	67.74±1.3	228.1±2.7
26	34	24	1	65.13±1.4	225.7±1.1
27	42	18	0.1	69.26±1.5	241.7±2.5

3^3 Full Factorial design was applied and various formulation batches were obtained by using Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA). Statistical analysis and optimization was performed using Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA) and JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK). Also, response surface graphs were generated using Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA) and Bubble plots using JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK). Obtained results were analyzed for statistical significance at 95% confidence interval (CI) and P value less than 0.05 was considered as statistically significant. Model terms were statistically analyzed by applying ANOVA, where model F value and p Value were used to determine the significance. Numerical optimization was done to obtain the optimized batch by setting the desired constraints of variables, where, the response variables fixed were maximum % EE and minimum spherulites size.

Effect of Independent variables on the dependent variables was studied using Response Surface Plots (3D plots) and Bubble plots. Obtained polynomial equation was used to study the quantitative effect of independent variables on dependent variables. Conclusion of optimization was drawn by studying desirability plots. Desirability ranges from 0 to 1, where

it shows confidence of a response to its ideal value. Total desirability is 1 when all the critical quality attributes achieve their ideal values.

5.3.1.3 Checkpoint Analysis

Polynomial equation and established plots were validated by performing a checkpoint analysis. Software suggested values of independent variables were taken and values of response variables were calculated by substituting the values in the polynomial equation. GCH loaded spherulites were prepared (N=3; ±SD) by taking optimized levels of independent variables (A, B and C). Predicted and experimentally obtained values of spherulites size and %EE were compared and their statistical significance was determined using 't' test.

5.3.2 Results and Discussion for Formulation and Optimization of GCH loaded Spherulites

Three independent variables viz. Phospholipid concentration (%w/w) (A), Hydration time (hrs) (B) and Probe-Cylinder distance (mm) (C) were varied at different levels in 3^3 Full Factorial design. Spherulites size and %EE were chosen as response variables. Total 27 batches were prepared and the results of the same have been given in Table 5.2. Table 5.2 represents the average result of variation in one variable at a time from its low to high level. The response variables values of spherulites i.e. %EE varied from 51.21 to 78.91% and spherulites size it varied from 200.2 to 252.3 nm.

5.3.2.1 Statistical evaluation of results of spherulites size

Spherulites size was statistically analyzed, where, all independent factors viz. Phospholipid concentration (%w/w) (A), Hydration time (hrs) (B) and Probe-Cylinder distance (mm) (C) showed that model terms were affecting the size of spherulites significantly. It was evidenced by statistical analysis at 95% CI, where p value (0.0001) obtained was less than 0.05. Variation in all three independent factors significantly affected the size of spherulites dispersion, which is depicted in Figure 5.3 with the help of a response surface graph.

After preparing all 27 batches from 3^3 Full Factorial design, regression analysis was done and a polynomial equation (eq.) was derived with the help of Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA).

$$Particle\ size = +204.78 + 0.19 * A + 0.89 * B - 7.85 * C - 1.15 * A * B + 1.62 * A * C - 1.45 * B * C + 1.70 * A^2 - 0.17 * B^2 + 30.65C^2 \quad \text{.....(eq.1)}$$

Where, A: Phospholipid concentration (%w/w),
B: Hydration time (hrs),
C: Probe-Cylinder distance (mm)

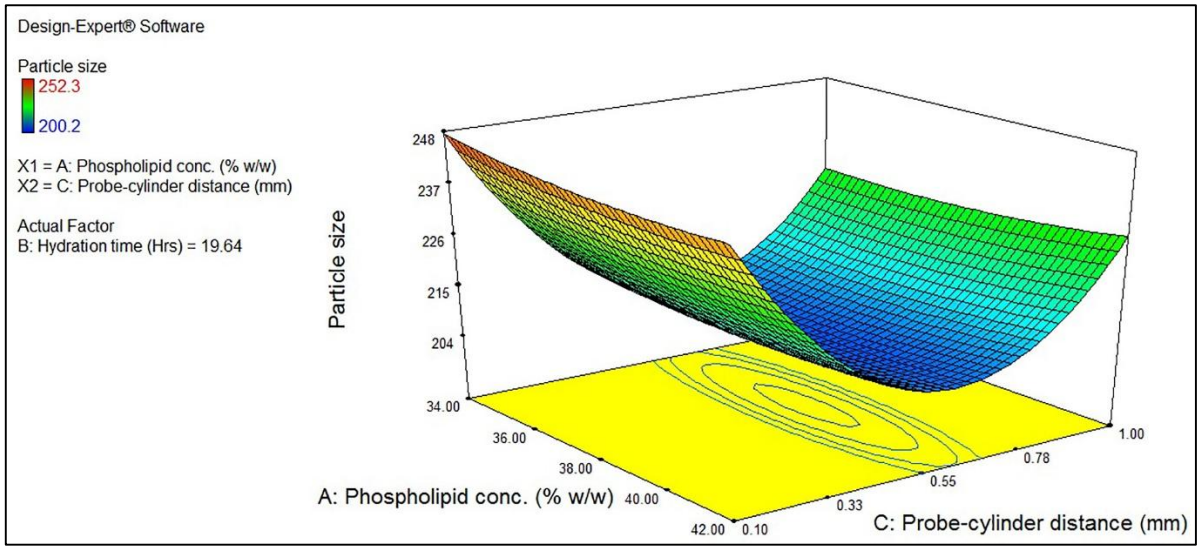

Figure 5.3: Response surface plot depicting the effect of independent variables on GCH loaded spherulites size.

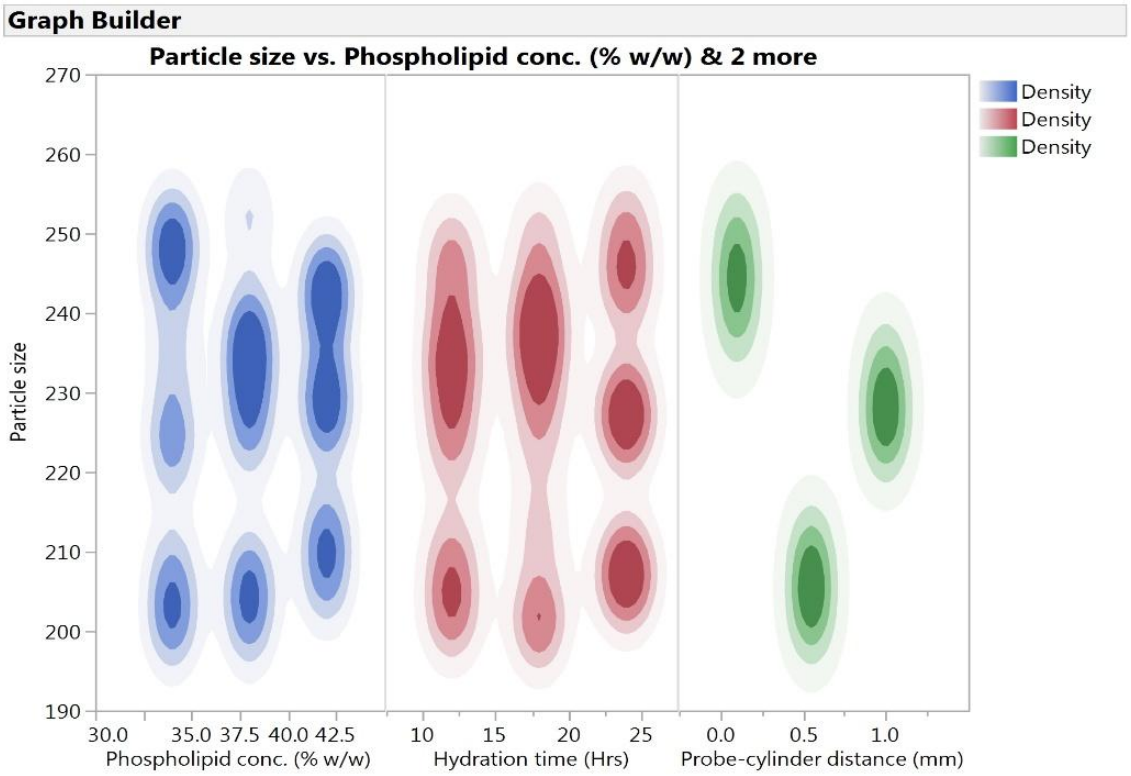

Figure 5.4: Bubble plot depicting the effect of independent variables on GCH loaded spherulites size.

A bubble plot showing the effect of independent factors over spherulites size was also plotted with the help of JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK) for a deeper insight. Figure 5.4 shows the bubble plot where all three independent factors are shown with their significant effect on particle size. The color coding helps to understand the impact of various levels of independent factors on response variable i.e. spherulites size.

Statistical determination of influence of independent variables over spherulites size was studied using the polynomial equation shown in eq.1. In the equation, positive sign before the values of coefficients A or B or C or AB or AC or BC or A^2 or B^2 or C^2 indicates that as the level of that particular factor is increased, the response value increases (here in particle size, the response value decreases, as we have kept criteria of minimum particle size) and vice versa for negative sign before coefficient. Here in eq.1 Factor A i.e. Phospholipid concentration affects the particle size as the level of factor was increasing, the particle size was decreasing which was desirable (100-750 nm) [8]. Moreover, it is reported that the selected range of phospholipid concentration yielded the spherulites of desired size range. However, reported literature stated that inclusion of a surfactant increases the particle size [9]. GCH loaded spherulites were prepared without inclusion of any surfactant [6, 7]. Factor B i.e. Hydration time (hrs) also influences positively, spherulites size, as longer hydration time yields better spherulites with even particle size distribution. This could be because, phospholipids gets self-assembled in an aqueous phase. However, excessive hydration could result into spherulites with discontinued bilayers [10]. Factor C i.e. Probe-Cylinder distance (mm) was observed to have highest effect. Where, the coefficient value for it was observed to be large. This indicates that as the variable of Factor C increases the particle size also increases. This effect is due to less shearing stress on the lamellar phase yielding larger particles. Moreover, it is reported that shearing of a lamellar phase affects the spherulites size [11]. Moreover, it is understood from polynomial equation (eq.1) that Factor A and C in combination influenced the particle size positively.

Analysis of Variance (ANOVA) was applied to reveal the statistical significance of the model. The Model F-value of 29.29 implies the model is significant. Values of "Prob > F" less than 0.05 indicate model terms are significant. Model p value was found to be < 0.0001, indicating the statistical significance.

5.3.2.2 Statistical evaluation of results of %EE

Statistical analysis of %EE was done, where, all independent factors viz. Phospholipid concentration (%w/w) (A), Hydration time (hrs) (B) and Probe-Cylinder distance (mm) (C) showed that model terms were affecting the %EE significantly. It is evidenced by statistical analysis at 95% CI, where p value (0.0089) obtained was less than 0.05. Variation in all three independent factors significantly affected the %EE of spherulites dispersion, which is depicted in Figure 5.5 with the help of a response surface graph.

After preparing all 27 batches of 3^3 Full Factorial design, regression analysis was done and a polynomial equation (eq.) was obtained with the help of Design Expert® (Version 7.0.0, State-

Ease Inc., Minneapolis, USA).

$$\%EE = +74.12 + 0.45 * A + 0.59 * B - 2.64 * C - 0.41 * A * B + 0.96 * A * C + 1.36 * B * C + 3.03 * A^2 - 0.88 * B^2 - 8.84 * C^2 \quad \text{(eq.2)}$$

Where, A: Phospholipid concentration (%w/w),
B: Hydration time (hrs),
C: Probe-Cylinder distance (mm)

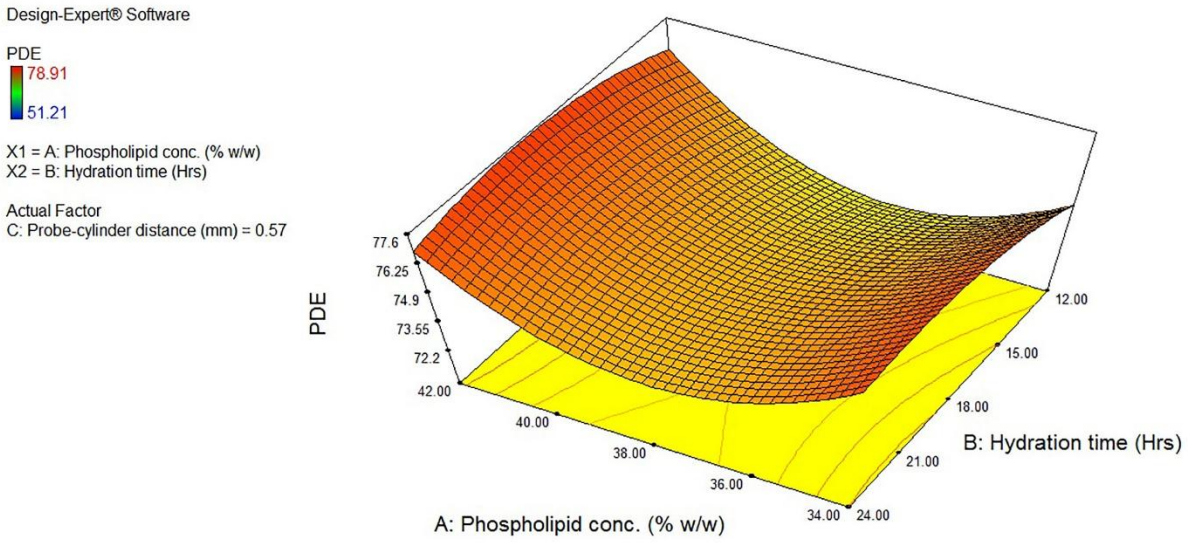

Figure 5.5: Response surface plot depicting the effect of independent variables on GCH loaded spherulites %EE.

A bubble plot showing the effect of independent factors over spherulites %EE was also plotted with the help of JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK) for more understanding. Figure 5.6 shows the bubble plot where all three independent factors are shown and their significant effect on %EE. The color coding helps to understand the impact of various levels of independent factors on response variable i.e. spherulites %EE.

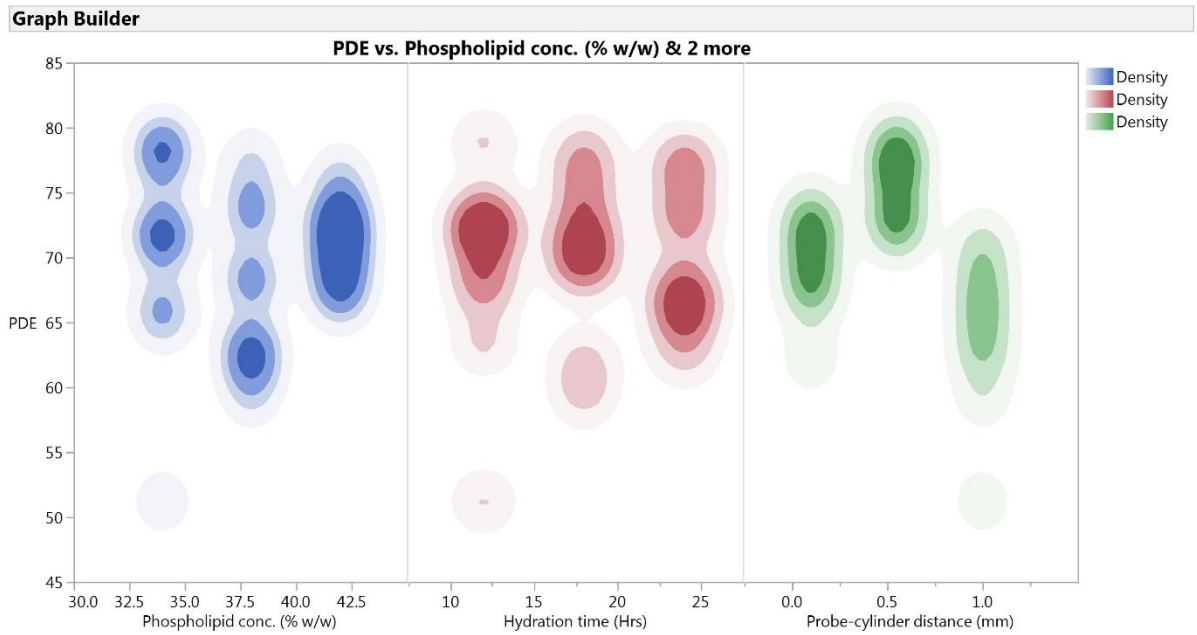

Figure 5.6: Bubble plot depicting the effect of independent variables on GCH loaded spherulites %EE.

Statistical determination of influence of independent variables over %EE was studied using the polynomial equation shown in eq.2. The positive sign before the values of coefficients A or B or C or AB or AC or BC or A^2 or B^2 or C^2 indicates that as the level of that particular factor increases, the response value increases (here in %EE the response value increases, as we have kept criteria of maximum %EE) and vice versa for negative sign before coefficient. Eq.1 suggested that Factor A i.e. Phospholipid concentration affects the %EE, as the level of factor was increasing, the %EE was increasing which was desirable. It was evidenced that the phospholipid concentration and %EE are linearly related i.e. as the concentration of lipid increases, %EE increases as well. As high concentration of phospholipid forms self-closed spherical particles which facilitates high %EE of drug [12]. Factor B i.e. Hydration time (hrs) also positively influences %EE, as longer hydration time yields maximum %EE of spherulites. Hydration time allows spherulites to form completely. Moreover, the mechanism of spherulites formation suggests that upon hydration the phospholipid bilayers grow from blisters to tubular fibrils and the water gets into the aqueous spaces of spherulites, this continues till the stabilization of bilayers into their equilibrium [13]. Factor C i.e. Probe-Cylinder distance (mm) was observed to have high value of coefficient. The negative sign before the coefficient value indicates that as the distance between probe-cylinder increases the %EE of spherulites decreases. Polynomial equation (eq.2) suggests that Factor A and C affected the %EE of spherulites in combination. Similarly, Factor B and C also affected the %EE.

Analysis of Variance (ANOVA) was applied to reveal the statistical significance of the model. The Model F-value of 3.77 implies the model is significant. Values of "Prob > F" less than 0.05 indicate model terms are significant. Model p value was found to be 0.0089, indicating the statistical significance.

Henceforth, desirability criteria was obtained using Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA). Desirability criteria was used to identify the optimized parameters for preparation of GCH loaded spherulites. Initially, acceptable criteria set were minimum particle size and maximum %EE. Consequently, by running all the batches of formulation, software offered optimized levels of all three independent factors viz. Factor A i.e. Phospholipid concentration (%w/w) was at 42% w/w, Factor B i.e. Hydration time (hrs) was at 19.66 hrs and Factor C i.e. Probe-Cylinder distance (mm) was at 0.57 mm. The calculated desirability factor i.e. the chance of obtaining the similar results offered by software was 0.915, which was found to be near to 1, indicating the suitability of the 3^3 Full Factorial design. Figure 5.7 shows the desirability plot of the optimization design.

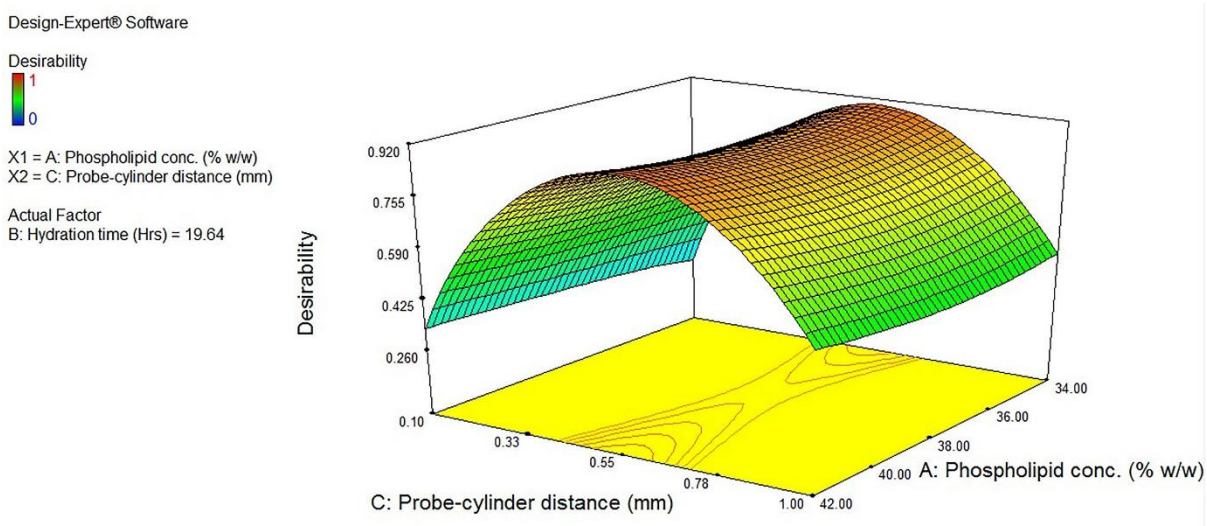

Figure 5.7: Desirability Plot for Optimization of GCH loaded spherulites.

5.3.2.3 Results of Checkpoint Analysis

A batch was prepared for the checkpoint analysis and characterized for both the response variables i.e. spherulites size and %EE (Table 5.3). Results indicated that the measured response was more accurately predicted by regression analysis that was proven by lower % Error value of regression analysis. Statistical significance was analyzed by preforming t test, where, no statistically significant difference (p value <0.05) was found between the predicted values and experimentally obtained values. Results have been shown in Table 5.3.

Table 5.3: Results of Checkpoint analysis of GCH loaded spherulites.

Response Parameters	Predicted value	Observed value[#]	Residual	% Error
Spherulites size (nm)	206.33	204.9±1.2	1.43	0.69
%EE	77.50	76.28±1.1	1.22	1.57

[#]Experiment was performed in triplicate (n=3; ±SD)

*Independent Factors: (A: 42%w/w; B: 19.66 hrs; C: 0.57 mm)

Statistical significance was determined by applying t test using GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, California, USA), where, p value obtained for spherulites size was 0.10 and for %EE was 0.12. Statistical t test revealed that no significant difference (p value >0.05) was found between the predicted values and experimentally observed values. This shows that the polynomial equation is validated.

Optimized batch of GCH loaded spherulites was PEGylated in order to improve the circulation time of the administered formulation *in vivo*. For that purpose DSPE-PEG 2000 (2 mole % of lipid phase) was used. DSPE-PEG 2000 was incorporated in the lipid phase of the formulation and remaining procedure was carried out in the same manner as described above (Section 5.3.1). The optimization of DSPE-PEG 2000 concentration in formulation has been discussed in previous Chapter 4-Preformulation studies. Final composition of Non-PEGylated and PEGylated GCH loaded spherulites is shown in Table 5.4.

Table 5.4: Composition of GCH loaded Non-PEGylated and PEGylated spherulites.

Formulation	Potassium Oleate	Cholesterol	SPC	DSPE-PEG 2000	Mannitol	Water	GCH
Non-PEGylated Spherulites	5 mg	20.9 mg	42 mg	-	3.5 mg	20 mL	10 mg
PEGylated Spherulites	5 mg	20.9 mg	42 mg	4 mg	3.5 mg	20 mL	10 mg

5.4 Preparation and Optimization of VLB loaded Spherulites

5.4.1 Preparation of spherulites formulation and optimization by Design of Experiment®

VLB loaded spherulites were prepared and optimized by applying Box-Behnken design. Spherulites were composed of SPC, Chol and Potassium oleate. Upon preliminary investigation (Chapter 4: Preformulation studies) independent variables were chosen viz. Phospholipid concentration (%w/w), Hydration time (hrs) and Probe-Cylinder distance (mm). While, spherulites size and %EE were response variables. Independent variables and their levels studied in Box-Behnken design are shown in Table 5.5. Figure 5.8 shows the

developmental process of VLB loaded Spherulites.

Table 5.5: Independent variables and their levels studied in Box-Behnken design.

Independent variables	Levels		
	Low (-1)	Intermediate (0)	High (+1)
Phospholipid concentration (%w/w)	34	38	42
Hydration time (hrs)	12	18	24
Probe-Cylinder distance (mm)	0.10	0.55	1.0

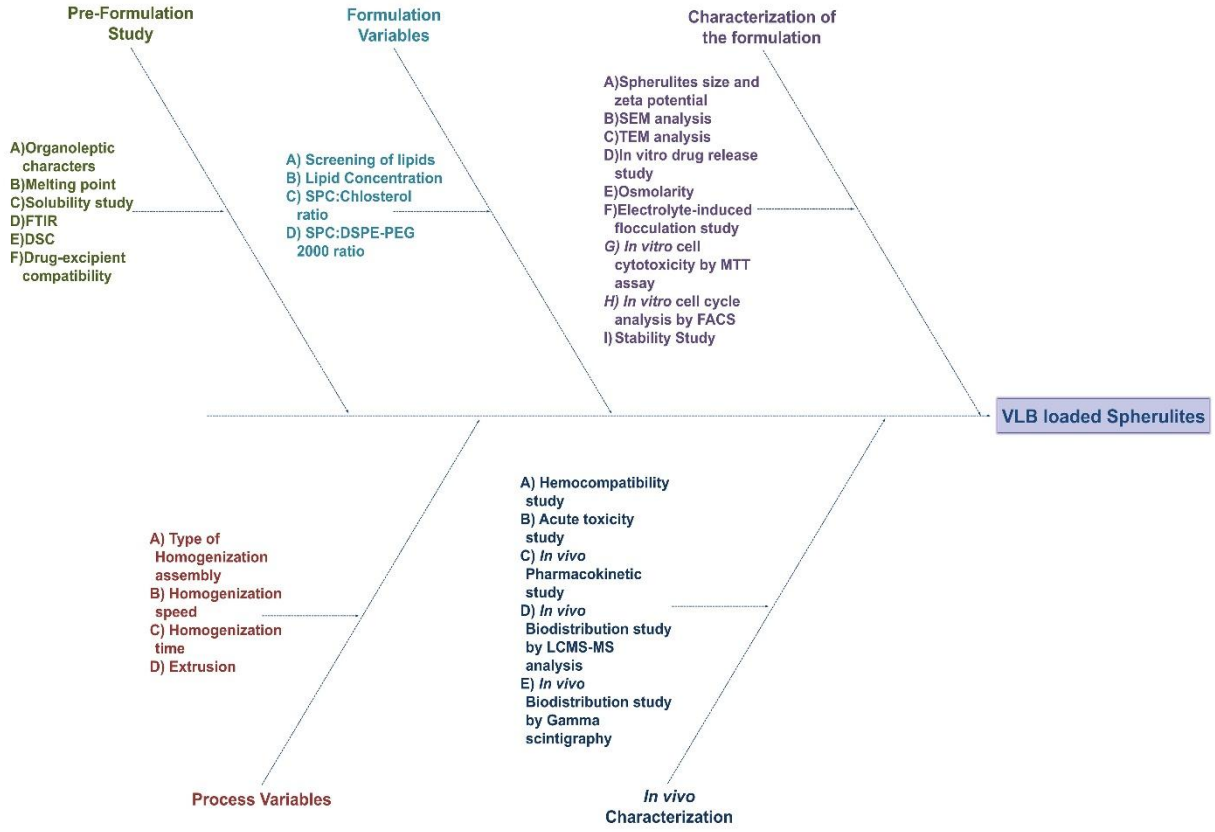

Figure 5.8: Ishikawa diagram showing the developmental process of VLB loaded Spherulites.

VLB loaded spherulites were prepared by patented procedure [6]. Briefly, lipid phase comprised of Soyabean Phosphatidylcholine (SPC)/Cholesterol (Chol) in 1:1M ratio, where, SPC and Chol were taken in round bottom flask (RBF) along with Potassium oleate. Lipid phase was dissolved in 2–3ml of chloroform: methanol mixture (9:1 v/v). The organic solvent mixture was evaporated by rotating RBF under vacuum and the obtained lipid film was dried at 60 °C for 15 min using a rotary evaporator (IKA RV10, Karnataka, India). The lipid film was dispersed using 20 ml of aqueous phase containing 10 mg VLB and Mannitol. The dispersion was allowed to hydrate overnight, followed by homogenization (Eurostar power control-visc, IKA, Bangalore, Karnataka, India) by employing an in house designed assembly of glass cylinder and teflon probe at controlled shear rate of 65 min^{-1} for 1 h. The sheared

dispersion was extruded 5 times through stainless steel syringe filter assembly comprising 0.22 µm filter [6, 14]. Figure 5.9 shows the flowchart of preparation procedure of GCH loaded spherulites.

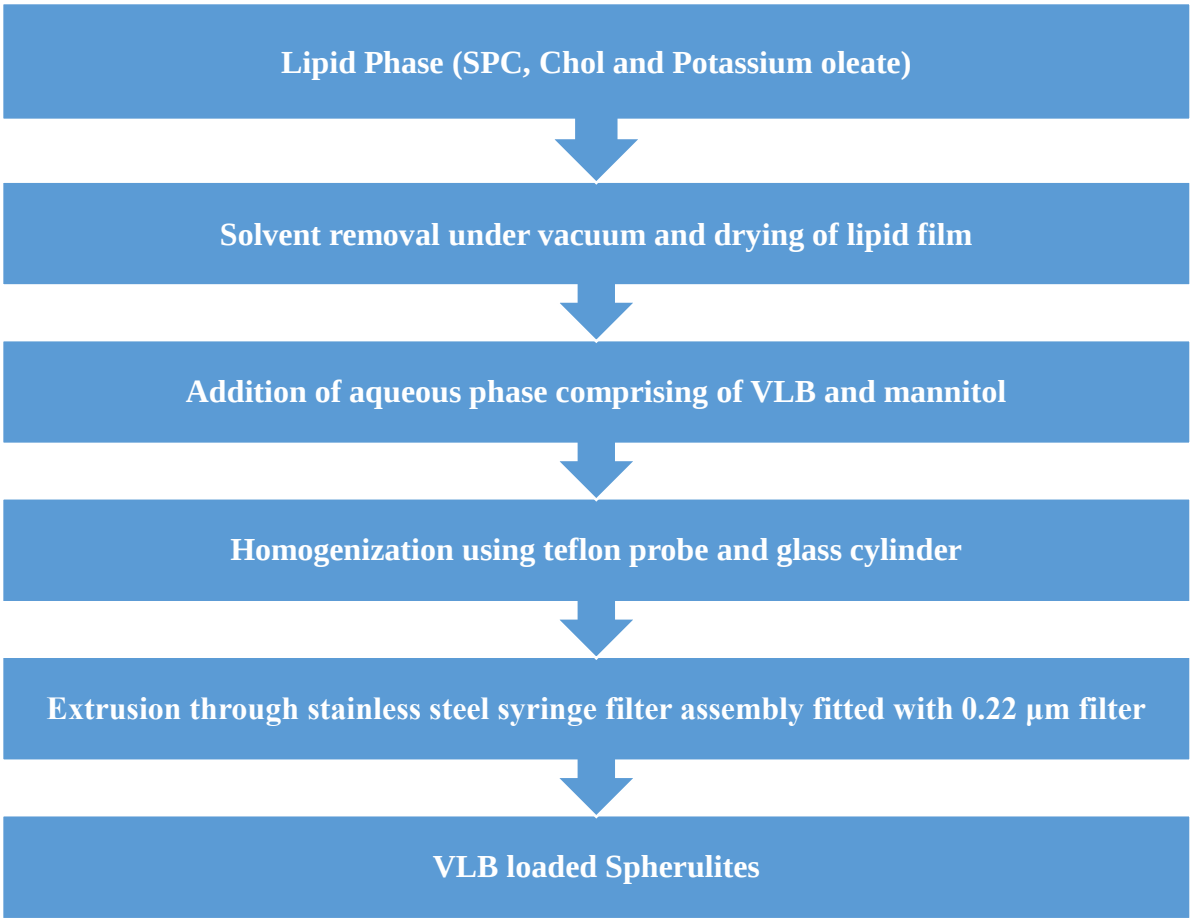

Figure 5.9: Flow chart depicting the preparation of VLB loaded Spherulites.

5.4.1.1 Determination of spherulites size

Briefly, 50 µL of dispersion was diluted with 2 ml of distilled water and taken in disposable polystyrene cuvette. Mean hydrodynamic diameter was measured in triplicate by dynamic light scattering using Malvern size analyzer (Malvern Nanoseries-ZS, Malvern Instruments, United Kingdom).

5.4.1.2 Determination of % Entrapment efficiency (% EE)

Spherulites loaded with VLB were taken in centrifuge tube and centrifuged at 20000 RPM at 4 ºC for 30 minutes (REMI Laboratory Instruments, Mumbai, India). Spherulites pellet settled at the bottom of tube was air dried and weighed for accounting the total weight of solid content. 2% Triton X100 solution was used to lyse the pellet and diluted suitably. Drug amount was estimated using UV spectrophotometer (Shimadzu 1800, Kyoto, Japan) at 271 nm for VLB.

Supernatant was also diluted suitably and free drug amount was estimated to establish the mass balance.

Table 5.6: Box-Behnken design batches with their measured responses (N=3; Mean±SD).

	Factor A	**Factor B**	**Factor C**	**Response 1**	**Response 2**
Batch	**Phospholipid conc. (% w/w)**	**Hydration time (Hrs)**	**Probe-cylinder distance (mm)**	**%EE (%)**	**Spherulites size (nm)**
1	38	18	0.55	93.20±1.3	180.7±2.2
2	42	12	0.55	96.20±1.1	129.5±1.9
3	42	18	0.1	88.60±1.6	126.7±2.7
4	34	12	0.55	95.70±1.2	156.2±2.1
5	38	24	1	94.80±1.4	417.4±2.4
6	34	18	1	93.82±1.2	373.6±1.6
7	34	18	0.1	87.60±1.8	120.1±2.1
8	38	24	0.1	87.60±1.6	210.6±1.8
9	38	18	0.55	93.20±1.4	180.7±1.4
10	42	18	1	94.6±1.5	452.8±2.3
11	38	12	0.1	88.42±1.4	126.4±2.1
12	42	24	0.55	93.00±1.3	126.7±1.2
13	38	18	0.55	93.20±1.9	180.7±1.6
14	38	12	1	94.40±1.1	407.2±2.5
15	38	18	0.55	93.20±1.4	180.7±2.3
16	38	18	0.55	93.20±1.1	180.7±1.9
17	34	24	0.55	95.20±1.5	131.6±2.1

Box-Behnken design was applied and various formulation batches were obtained by using Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA). Statistical analysis and optimization was performed using Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA) and JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK). Also, response surface graphs were generated using Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA) and Bubble plots using JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK). Obtained results were analyzed for statistical significance at 95% confidence interval (CI) and P value less than 0.05 was considered as

statistically significant. Model terms were statistically analyzed by applying ANOVA, where model F value and p Value were used to determine the significance. Numerical optimization was done to obtain the optimized batch by setting the desired constraints of variables, where, the response variables were fixed as maximum % EE and minimum spherulites size.

Effect of Independent variables over dependent variables was studied using Response Surface Plots (3D plots) and Bubble plots. Obtained polynomial equation was used to study the quantitative effect of independent variables on dependent variables. Conclusion of optimization was drawn by studying desirability plots. Desirability ranges from 0 to 1, where it shows closeness of a response to its ideal value. Total desirability is 1 when all the critical quality parameters achieve their ideal values.

5.4.1.3 Checkpoint Analysis

Polynomial equation and established plots were validated by performing a checkpoint analysis. Software suggested values of independent variables were taken and values of response variables were calculated by substituting the values in the polynomial equation. VLB loaded spherulites were prepared (N=3; ±SD) by taking optimized levels of independent variables (A, B and C). Predicted and experimentally obtained values of spherulites size and %EE were compared and their statistical significance was determined using 't' test.

5.4.2 Results and Discussion for Formulation and Optimization of VLB loaded Spherulites

Three independent variables viz. Phospholipid concentration (%w/w) (A), Hydration time (hrs) (B) and Probe-Cylinder distance (mm) (C) were varied at different levels in Box-Behnken design. Spherulites size and %EE were chosen as response variables. Total 17 batches were prepared experimentally and the results of the same has been given in Table 5.6. Table 5.6 represents the average result of variation in one variable at a time from its low to high level. The response variables values for spherulites %EE varied from 87.60 to 96.2%, similarly, for spherulites size it varied from 120.1 to 452.8 nm.

5.4.2.1 Statistical evaluation of results of spherulites size

Spherulites size was statistically analyzed, where, all independent factors viz. Phospholipid concentration (%w/w) (A), Hydration time (hrs) (B) and Probe-Cylinder distance (mm) (C) showed that model terms were affecting the size of spherulites significantly. It was evidenced by statistical analysis at 95% CI, where p value (<0.0001) obtained was less than 0.05. Variation in all three independent factors significantly affected the size of spherulites

dispersion, which is depicted in Figure 5.10 with the help of a response surface graph.

After preparing all 17 batches of Box-Behnken design, regression analysis was done and a polynomial equation (eq.) was obtained with the help of Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA).

$$Particle\ size = +180.70 + 6.78 * A + 8.38 * B + 133.40 * C + 5.45 * A * B + 18.15 * A * C - 18.50 * B * C - 33.40 * A^2 - 11.30 * B^2 + 121.00 * C^2 \ldots.(eq.3)$$

Where, A: Phospholipid concentration (%w/w),
B: Hydration time (hrs),
C: Probe-Cylinder distance (mm)

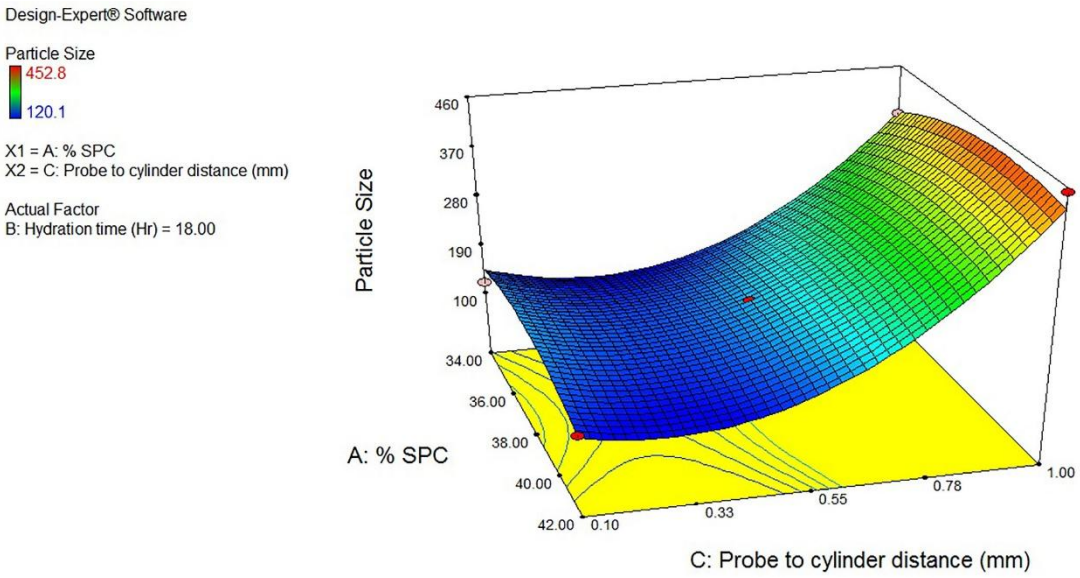

Figure 5.10: Response surface plot depicting the effect of independent variables on VLB loaded spherulites size.

A bubble plot showing the effect of independent factors over spherulites size was also plotted with the help of JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK) for more understanding. Figure 5.11 shows the bubble plot where all three independent factors are shown with their significant effect on particle size. The color coding helps to understand the impact of various levels of independent factors on response variable i.e. spherulites size.

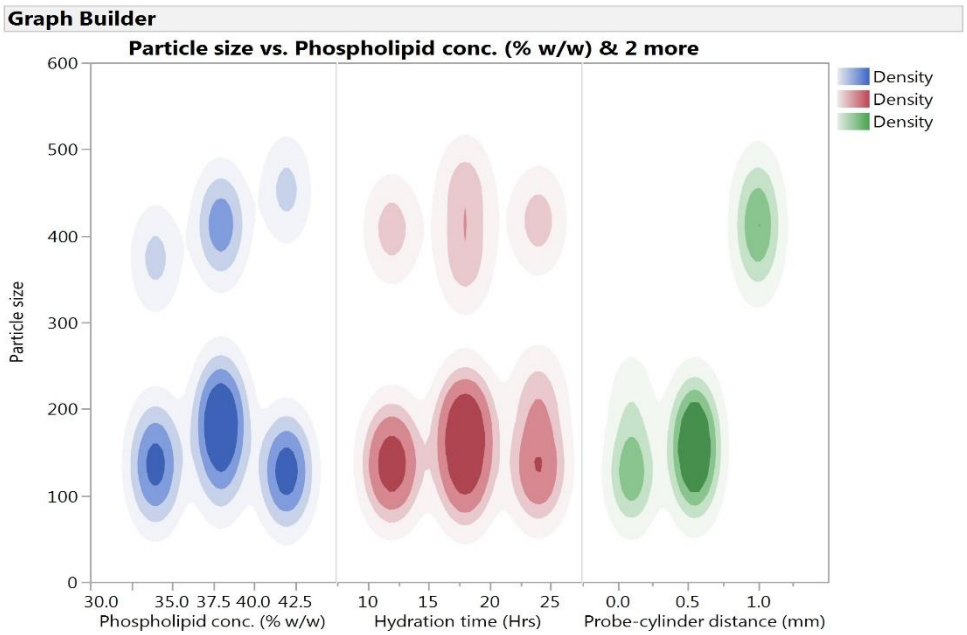

Figure 5.11: Bubble plot depicting the effect of independent variables on VLB loaded spherulites size.

Statistical determination for influence of independent variables over spherulites size was studied using the polynomial equation shown in eq.3. The positive sign before the values of coefficients A or B or C or AB or AC or BC or A^2 or B^2 or C^2 indicates that as the level of that particular factor is increased, the response value increases (here in particle size the response value decreases, as we have kept criteria of minimum particle size) and vice versa for negative sign before coefficient. After studying Eq.3, it was revealed that Factor A i.e. Phospholipid concentration was found to be affecting the size of spherulites at different levels. The positive sign before the coefficient of Factor A indicates that as the level of phospholipid concentration increases, spherulites were formed i.e. high concentration of lipid yielded better vesicles. It was evidenced that certain range of phospholipid concentration effectively yield the spherulites of desired size. Previously reported work included non-ionic surfactants in formulation composition. In present investigation VLB loaded spherulites were prepared without inclusion of any surfactant [6, 14]. Factor B i.e. Hydration time (hrs) was also found to affect the size. High value of coefficient with positive sign indicated that, hydration of lipid phase for optimum time yields better spherulites with even particle size distribution. This could be because phospholipids gets self-assembled in an aqueous phase. However, excessive hydration could result into spherulites with discontinued bilayers which results into premature drug release or leaching of drug from the carrier [10]. Factor C i.e. Probe-Cylinder distance (mm) was observed to be affecting the spherulites size as well. Shearing of lamellar phase i.e. hydrated

lipid phase is nothing but the grinding, which effectively reduces the size of particles, moreover, it improves the bilayers integrity. The orientation gap between the glass tube and teflon probe effectively define the size as the lipid phase flows in one direction while shearing [15, 16]. Moreover, it was observed from polynomial equation (eq.3) that Factor A and B and Factor A and C in combination influenced the particle size positively.

Analysis of Variance (ANOVA) was applied to reveal the statistical significance of the model. The Model F-value of 35.41 implies the model is significant. Values of "Prob > F" less than 0.05 indicate model terms are significant. Model p value was found to be < 0.0001, indicating the statistical significance.

5.4.2.2 Statistical evaluation of results of %EE

Statistical analysis of %EE was analyzed, where, all independent factors viz. Phospholipid concentration (%w/w) (A), Hydration time (hrs) (B) and Probe-Cylinder distance (mm) (C) showed that model terms were affecting the %EE significantly. It was evidenced by statistical analysis at 95% CI, where p value (<0.0001) obtained was less than 0.05. Variation in all three independent factors significantly affected the %EE of spherulites dispersion, which is depicted in Figure 5.12 with the help of a response surface graph.

After preparing all 17 batches of Box-Behnken design, regression analysis was done and a polynomial equation (eq.) was obtained with the help of Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA).

$$\%EE = +93.20 + 0.01 * A - 0.51 * B + 3.18 * C - 0.68 * A * B - 0.05 * A * C + 0.31 * B * C + 0.84 * A^2 + 0.99 * B^2 - 2.88 * C^2 \quad \text{(eq.4)}$$

Where, A: Phospholipid concentration (%w/w),
B: Hydration time (hrs),
C: Probe-Cylinder distance (mm)

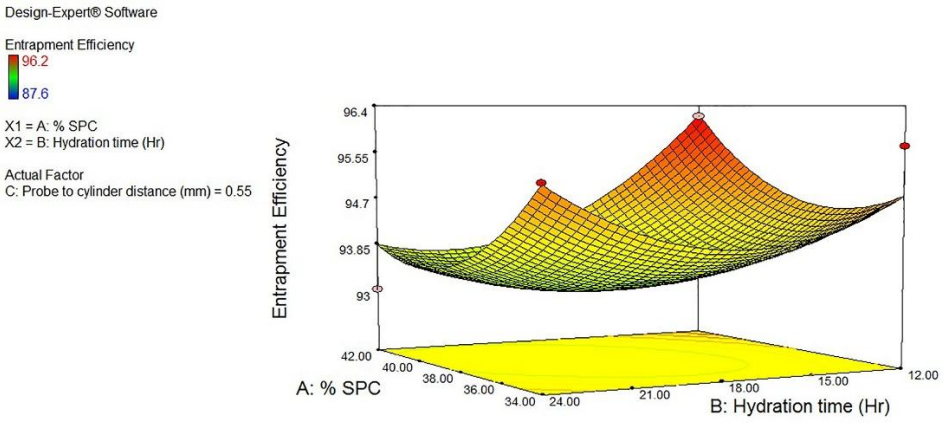

Figure 5.12: Response surface plot depicting the effect of independent variables on VLB loaded spherulites %EE.

A bubble plot showing the effect of independent factors over spherulites %EE was also plotted with the help of JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK) for more understanding. Figure 5.13 shows the bubble plot where all three independent factors are shown with their significant effect on %EE. The color coding helps to understand the impact of various levels of independent factors on response variable i.e. spherulites %EE.

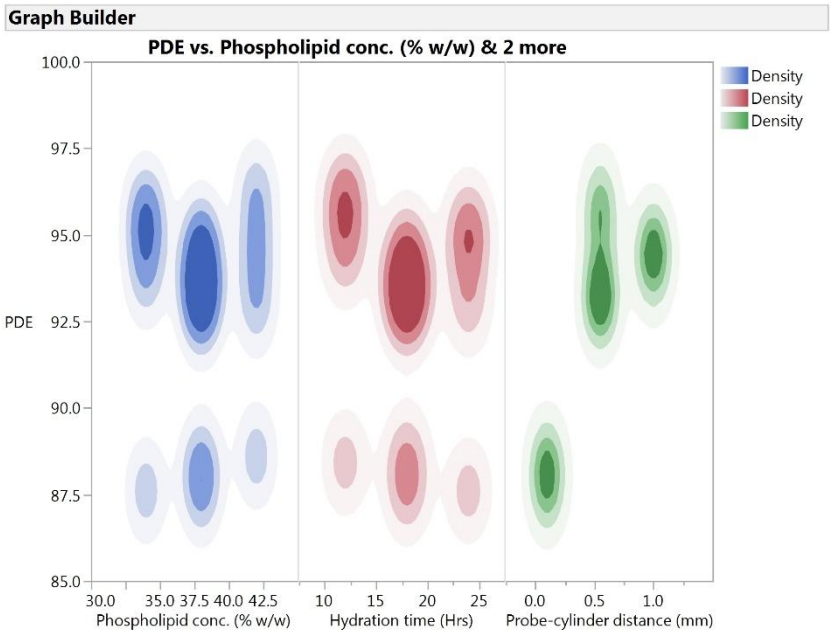

Figure 5.13: Bubble plot depicting the effect of independent variables on VLB loaded spherulites %EE.

Statistical determination of influence of independent variables over spherulites %EE was studied using the polynomial equation shown in eq.4. The equation can understood as positive sign before the values of coefficients A or B or C or AB or AC or BC or A^2 or B^2 or C^2 indicates that as the level of that particular factor is increased, the response value increases (here in %EE the response value increases, as we have kept criteria of maximum %EE) and vice versa for negative sign before coefficient. Eq.4 suggested that Factor A i.e. Phospholipid concentration affects the %EE as the level of factor was increasing, the %EE was increasing which was desirable. It was evidenced that the phospholipid concentration and %EE are linearly related i.e. as the concentration of lipid increases, %EE increases as well. As high concentration of phospholipid forms self-closed spherical particles which facilitates high %EE of drug [12]. However, Factor B i.e. Hydration time (hrs) was observed to have negative influence on %EE, as it suggested that longer hydration of lipid film yielded spherulites with less entrapment of drug. It is reported that optimum hydration time allows spherulites to form completely. Moreover, the mechanism of spherulites formation suggest that upon hydration the

phospholipid bilayer grow from blisters to tubular fibrils and the water gets into the aqueous spaces of spherulites, this continues till the stabilization of bilayers into their equilibrium [13]. Factor C i.e. Probe-Cylinder distance (mm) affected the %EE of spherulites. It was evidenced that the shearing rate (discussed in Chapter 4. Preformulation studies) and shearing assembly affects the encapsulation of drug in spherulites. Where, during shearing with the glass tube and teflon probe, lipid phase flows in a Newtonian way. However, large gap between the glass tube and teflon probe result into loose shearing yielding less %EE [11, 16]. Polynomial equation (eq.4) suggests that Factor B and C affected the %EE of spherulites in combination.

Analysis of Variance (ANOVA) was applied to reveal the statistical significance of the model. The Model F-value of 32.74 implies the model is significant. Values of "Prob > F" less than 0.05 indicate model terms are significant. Model p value was found to be < 0.0001, indicating the statistical significance.

Henceforth, desirability criteria was obtained using Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA). Desirability criteria was used to identify the optimized parameters for preparation of VLB loaded spherulites. Initially, acceptable criteria set were minimum particle size and maximum %EE. Consequently, by running all the batches of formulation software offered optimized levels of all three independent factors viz. Factor A i.e. Phospholipid concentration (%w/w) was at 42% w/w, Factor B i.e. Hydration time (hrs) was at 12 hrs and Factor C i.e. Probe-Cylinder distance (mm) was at 0.53 mm. The calculated desirability factor i.e. the chance of obtaining the similar results offered by software was 0.992, which was found to be near to 1, indicating the suitability of the Box-Behnken design. Figure 5.14 has been shown with the desirability plot of the optimization design.

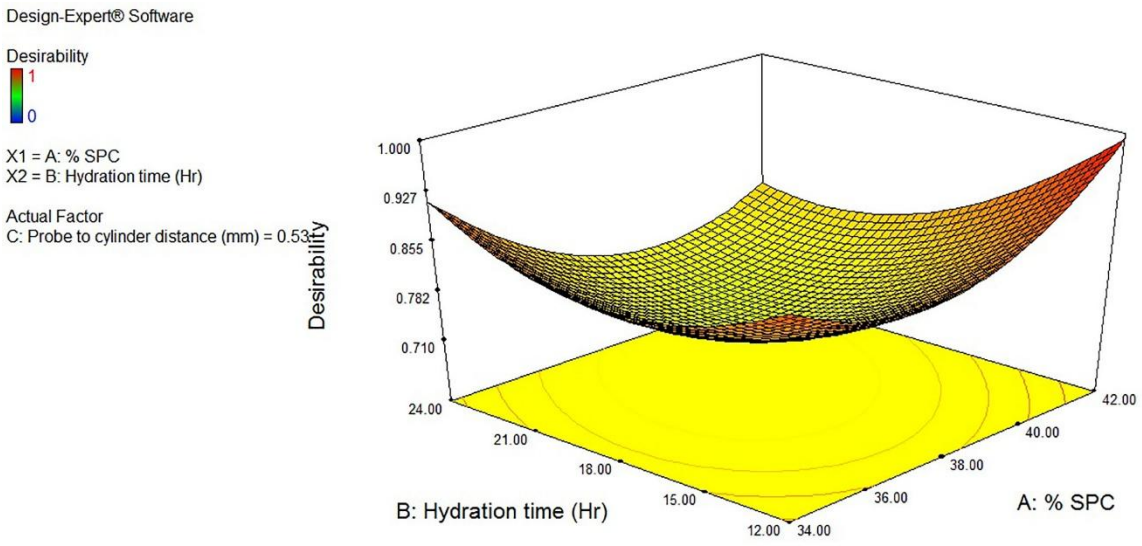

Figure 5.14: Desirability Plot for Optimization of VLB loaded spherulites.

5.4.2.3 Results of Checkpoint Analysis

A batch was prepared for the checkpoint analysis and characterized for both response variables i.e. spherulites size and %EE (Table 5.7). Results indicated that the measured response was more accurately predicted by regression analysis that was proven by lower % Error value of regression analysis. Statistical significance was analyzed by preforming t test, where, no statistically significant difference (p value <0.05) was found between the predicted values and experimentally obtained values. Results have been shown in Table 5.7.

Table 5.7: Results of Checkpoint analysis of VLB loaded spherulites.

Response Parameters	Predicted value	Observed value[#]	Residual	% Error
Spherulites size (nm)	120.1	122.4±1.6	2.3	1.91
%EE	96.06	95.65±0.86	0.41	0.42

[#]Experiment was performed in triplicate (n=3; ±SD)

*Independent Factors: (A: 42%w/w; B: 12 hrs; C: 0.53 mm)

Statistical significance was determined by applying t test using GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, California, USA), where, p value obtained for spherulites size was 0.067 and for %EE was 0.45. Statistical t test revealed that no significant difference (p value >0.05) was found between the predicted values and experimentally observed values. This shows that the polynomial equation is validated.

Optimized batch of VLB loaded spherulites was PEGylated in order to improve the circulation time of the administered formulation *in vivo*. For that purpose DSPE-PEG 2000 (2 mole % of lipid phase) was used. DSPE-PEG 2000 was incorporated in the lipid phase of the formulation and remaining procedure was carried out in the same manner as described above (Section 5.3.1). The optimization of DSPE-PEG 2000 concentration in formulation has been discussed in previous Chapter 4. Preformulation studies. Final composition of Non-PEGylated and PEGylated VLB loaded spherulites has been shown in Table 5.8.

Table 5.8: Composition of VLB loaded Non-PEGylated and PEGylated spherulites.

Formulation	Potassium Oleate	Cholesterol	SPC	DSPE-PEG 2000	Mannitol	Water	VLB
Non-PEGylated Spherulites	5 mg	20.9 mg	42 mg	-	3.5 mg	20 mL	10 mg
PEGylated Spherulites	5 mg	20.9 mg	42 mg	4 mg	3.5 mg	20 mL	10 mg

5.5 References

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