Chapter 5

FORMULATION

DEVELOPMENT

5.1 Introduction

Quality has been given an importance by all regulatory bodies for pharmaceutical products. Quality means customer satisfaction in terms of service, product, and process. Quality, productivity, cost, time and value are interrelated terms. Hence the quality has to be built in the product through proper planning, so that the forth coming failure can be avoided. Mere analysis of final product will not work but the quality should be built in the product. The principles of Quality by Design (QbD) have been used to advance the product and process quality in every industry. [1] This concept was first outlined by well-known quality expert Joseph M. Juran. It means designing and developing formulations and manufacturing processes to ensure predefined product quality objectives.

One of the tools for building quality in the product is through Design of experiments. Design of experiments (DOE) is a structured and organized method to determine the relationship among factors that influence outputs of a process. It has been suggested that DOE can offer returns that are four to eight times greater than the cost of running the experiments in a fraction of the time. Application of DOE in QbD helps in gaining maximum information from a minimum number of experiments. [2] Optimization of any pharmaceutical process begins with the objectives to find out and evaluate independent variables that affect formulation response, determine them and establish their best response values. While developing a pharmaceutical product, various formulations as well as process variables related to effectiveness, safety and usefulness should be simultaneously optimized. Polynomial non-linear regression analysis is widely used for establishing approximate mathematical models in which the variables are screened by stepwise selection method according to statistical significance [3, 4] and final model would be used to predict the relationship between different variables and their levels. Optimization by changing one-variable-at-a-time is a complex method to evaluate the effects of different variables on an experimental outcome. Another approach is to accurately evaluate the impact of the independent variables on the dependent variables by varying all the important factors simultaneously in a systematic manner. This approach is known as response surface methodology (RSM). RSM is a statistical technique, which can address the present scenario and can be used to establish relationships between

several independent variables and one or more dependent variables [5]. RSM optimizes multiple variables by systematic variation of all variables in a well-designed experiment with a minimum number of experiments. The RSM optimization process involves the following steps: performing statistically designed experiments; estimating the coefficients of a mathematical model using regression analysis technique; and predicting the response and checking the adequacy of the model. Among the available statistical design methods, a full factorial design measures the response of every possible combination of factors and factor levels. These responses are analyzed to provide information about every main effect and every interaction effect. However, a full factorial DOE is only practical when fewer than five factors are being investigated. Testing all combinations of factor levels becomes too expensive and time-consuming with five or more factors. [5]

In the present investigation, among the various independent factors studied during the preliminary trials, which could affect the size of vesicles and entrapment efficiency of formulation, Lipid: Cholesterol ratio and hydration time were found to have a significant effect on the responses measured. Since, we analyzed two critical factors (Lipid: Cholesterol and Hydration time) affecting size of vesicles and % EE, hence, 3^2 full factorial design was chosen to optimize the liposomal formulations. In a 3^2 full factorial design, each experimental factor has three levels i.e. low (-1), intermediate (0) and high (+1). Hence there will be total 9 runs of the experiment with all the possible combinations of factor levels. Based upon the polynomial equation generated by model fitting and optimization, the effect of various independent factors can be analyzed on the responses measured (here, size and % EE).

5.2 Materials and Equipment

Materials

Leuprolide acetate was obtained as a gift sample from Sun Pharma Advanced Research Centre, Vadodara, India and Raloxifene Hydrochloride was obtained as a gift sample from Aarti drugs Ltd. Mumbai, India. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was obtained as gift sample from Avanti Polar Lipids, Inc. Alabaster, Alabama. Cholesterol was purchased from Sigma Aldrich, Bangalore, India. Methanol and Chloroform (A. R. grade) was purchased from Merck, Mumbai, India. Distilled water

used in the study was filtered using 0.22 micron nylon filter, Nylon N66 membrane filters 47 mm, Rankem, India. D- Mannitol and Gelatin were purchased from Sigma Aldrich, UK. Silicone tubing of 2x4mm was purchased from VWR International, UK. All other reagents were purchased from S.D. fine-chem limited, Baroda, India and were of analytical grade.

Equipment

- 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)
- Probe Sonicator (LabsonicM, Sartorius Ltd, Mumbai, India)
- Programmable freeze dryer (VirTis Advantage, SP Scientific, UK)

5.3 Preparation and Optimization of Raloxifene Hydrochloride loaded Liposomes

5.3.1 Preparation of liposomal formulation and optimization by Design of Experiment

3^2 full factorial design was applied to prepare RLX loaded liposomes using DSPC and Cholesterol. Independent variables chosen were Lipid: Cholesterol ratio (X) and Hydration time (Y), while dependent variables were vesicle size (nm) and % Entrapment Efficiency. Table 5.1 shows the independent factors and their levels studied in 3^2 full factorial design.

Table 5.1 Factors and Levels of factors studied in the design

Independent Variables	Low (-1)	Intermediate (0)	High (+)
Lipid: Cholesterol	1:1	2:1	3:1
Hydration time (hours)	0.5	1	1.5

Briefly, Stock solution of DSPC and Cholesterol was prepared in Chloroform: Methanol mixture (9:1). For it, 20 mg each of DSPC and Cholesterol was weighed individually and transferred to small glass vials. To it 2 ml of Chloroform: Methanol mixture was added and the contents were shaken properly on vortex mixer. DSPC, Cholesterol and 6 mg

drug were taken in Round Bottom Flask (RBF). RBF was then attached to rotary evaporator with temperature maintained at 60 ºC for 20 minutes at 120 rpm. After the thin lipid film forms, RBF was removed and nitrogen flushing was done to remove traces of organic solvent. Hydration of lipid film was done using distilled water at 60 ºC. The size of liposomes was then reduced by probe sonication 3 cycles of one minute at amplitude of 60 %, 0.6 s (MSE Soniprep, 150 Plus, London, UK).

5.3.1.1 Determination of Vesicle size: The Vesicle size and size distribution of formulations were determined by using Dynamic light scattering using Malvern Zetasizer (NanoZS, Malvern Instruments, UK). 50 µL of liposomal dispersion was added to 2 ml distilled water and it was then analyzed for Vesicle Size and size distribution.

5.3.1.2 Determination of % Entrapment Efficiency: Prepared liposomal dispersions were taken in eppendorf tubes and centrifuged (REMI Laboratory Instruments, Mumbai, India) at 20,000 rpm at 4 ºC for 30 minutes. The liposomal pellet settles down while free drug remains in supernatant. The liposomal pellet was air-dried and lysed using methanol. The contents were appropriately diluted and analyzed using UV spectrophotometry at 287 nm. To confirm the mass balance, free drug present in supernatant was also analyzed by UV-Spectrophotometry. 0.1 ml of supernatant was taken and diluted appropriately and absorbance of resultant solution was measured at 287 nm. % EE was calculated using the formula given below:

$$\% \text{ EE } = \frac{\text{Estimated Entrapped drug in Liposomes}}{\text{Total drug added to formulation}} \times 100$$

Experimental batches with the measured responses have been given in Table 5.2.

Table 5.2 3^2 Full Factorial experimental layout with the measured responses

Batch	X	Y	Size (nm)*	% EE*
1	1:1	0.5	125±1.4	76.83±1.1
2	1:1	1	207±1.0	77.26±1.2
3	1:1	1.5	119±2.1	77.68±1.5
4	2:1	0.5	119.3±1.3	87.45±2.1
5	2:1	1	118.7±1.1	88.6±2.2
6	2:1	1.5	122.1±1.2	90.96±1.4
7	3:1	0.5	115.5±1.5	91.0±1.1
8	3:1	1	130.2±1.2	89.98±1.5
9	3:1	1.5	110±2.2	89.96±1.3

*Experiment was done in triplicate (n=3)

Statistical analysis of the experimental data and optimization of the formulation was done using Design Expert software (Version 9.0.0.7, State-Ease Inc., Minneapolis, USA) and JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK). Results obtained were statistically analyzed at 5% level of significance and best fitting model was identified. Based on p value obtained conclusion was drawn whether the model terms are significant or non significant. P value less than 0.05 was considered statistically significant. ANOVA was also applied to test the significance of model terms. Model F value and p value were used to conclude the result. Numerical optimization was performed using this software by setting the desired constraints for various variables to obtain the optimized batch with desired responses. Our optimization criteria/constraints included maximum % EE and minimum vesicular size.

With the help of Response Surface Plots (3D plots), and Bubble plots conclusion was drawn regarding the effect of independent variables on dependent variables. Based upon the polynomial equation generated, the quantitative effect of independent factors was studied on the response values. Over all desirability can be obtained from desirability plots of optimization study. The desirability lies between 0 and 1 and it represents the closeness of a response to its ideal value. If both the quality characteristics reach their ideal values, the individual desirability is 1 for both. Consequently, the total desirability is also 1.

5.3.1.3 Checkpoint Analysis

A checkpoint analysis was performed to confirm the utility of the established plots and polynomial equation in the preparation of liposomes. Values of independent variables (X and Y) were taken from solutions suggested by the software and the values of % EE and vesicle size were calculated by substituting the values in the polynomial equation. RLX loaded liposomes were prepared experimentally by taking the levels of the independent variables (X and Y). Each batch was prepared three times and mean values were determined. Difference in the predicted and mean values of experimentally obtained % EE and vesicle size was compared by using statistical 't' test.

5.3.2 Results and Discussion for Formulation and Optimization of RLX-Loaded Liposomes

Nine batches of RLX loaded liposomes were prepared using 3^2 full factorial design varying two independent factors viz. Lipid: Cholesterol ratio (X) and Hydration time (Y). Vesicle size (nm) and % EE were taken as dependent variables. The results of the same have been shown in Table 5.2. The main effects of X and Y represent the average result of changing one variable at a time from its low to high value. The values for the nine batches varied from 76.83 to 90.96 % and 110 to 207 nm for % EE and vesicle size respectively.

5.3.2.1 Statistical evaluation of results of vesicle size

When the results of vesicle size were analyzed statistically using a 5% level of significance, p value obtained was more than 0.05, indicating the model terms are insignificant. This means that Lipid: Cholesterol ratio and Hydration time had no significant effect on vesicle size. Varying the factor X or Y did not produce any significant variation in the size of liposomal dispersion. The same can be explained with the help of Response Surface plot (Figure 5.1).

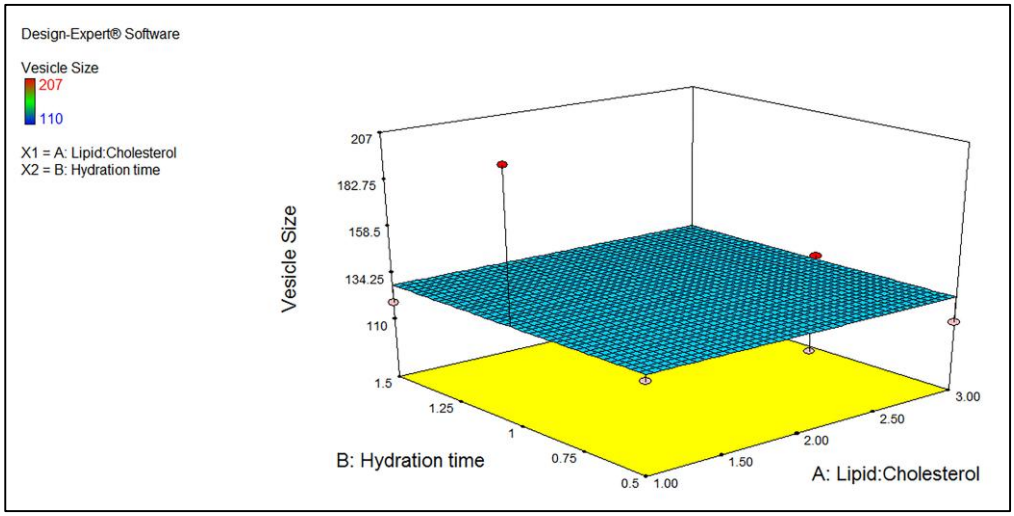

Figure 5.1 Response Surface Plot for showing the effect of independent variables on Vesicle size of RLX-Liposomes

Figure 5.2 shows the bubble plot, which also explains that as the Lipid: Cholesterol ratio is increased the vesicle size remains insignificantly affected. Similarly, increasing the hydration time from 0.5 hours to 1.5 hours has no change in the size.

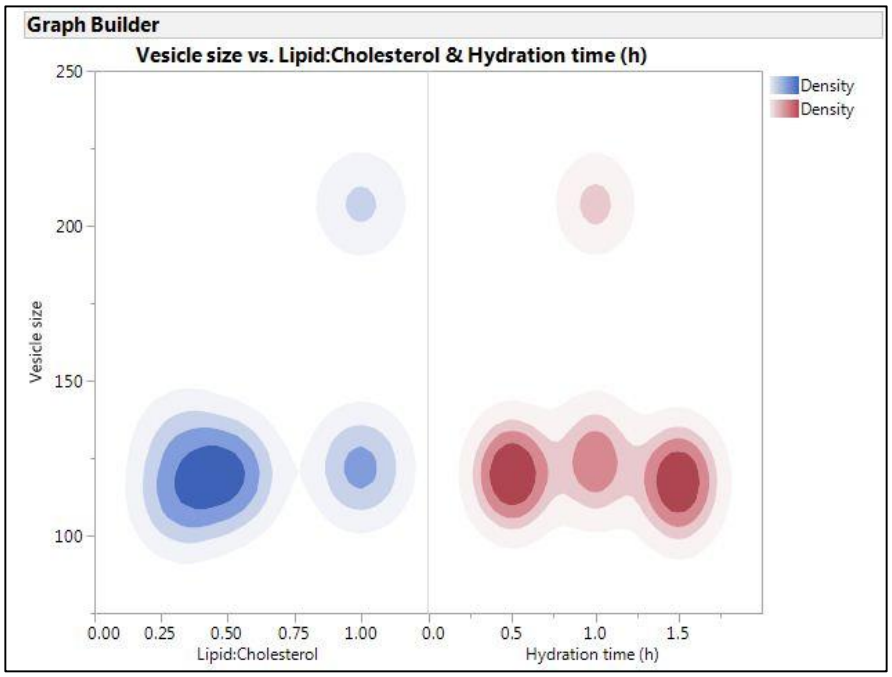

Figure 5.2 Bubble plot to study the effect of independent factors on vesicle size of RLX-Liposomes (In this figure 0.3 represents 3:1, 0.5 represents 2:1 and 1 represents 1:1 Lipid: Cholesterol ratio on X axis)

5.3.2.2 Statistical evaluation of results of % EE

When the results of % EE were analyzed statistically using a 5% level of significance, p value (0.0008) obtained was less than 0.05, indicating the model terms are significant. This means that Lipid: Cholesterol ratio and Hydration time had a significant effect on entrapment efficiency of RLX into the liposomes.

The polynomial equation obtained after regression analysis using the software Design Expert (Version 9.0.0.7, State-Ease Inc., Minneapolis, USA) was:

% EE= +88.70+6.53*A (p value 0.0001)+0.55*B-0.47*A*B-5.18*A^2 (p value 0.0017)+0.40*B^2

where, A represents Lipid: Cholesterol ratio and B represents Hydration time (hours)

Positive sign before the coefficient values of A or B or AB in the equation represents that as the value of that particular factor is increased, the response value increases and vice-versa. Whereas, negative sign indicates the decrease in the response value with increase in the value of independent factor. Also the value of the coefficient before the factor in the equation shows how significantly that factor affect the measured response compared to other factor. In this case, A, A^2 are significant model terms having p value less than 0.1 and having higher coefficient values than B, AB or B^2.

Analysis of Variance (ANOVA) was applied for estimation of significance of the model using a 5% significance level. The Model F-value of 57.31 implies that the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case Prob > F was 0.0008 indicating the significant effect of Lipid: Cholesterol and Hydration time on % EE. The same can be explained using the bubble plot given in Figure 5.3.

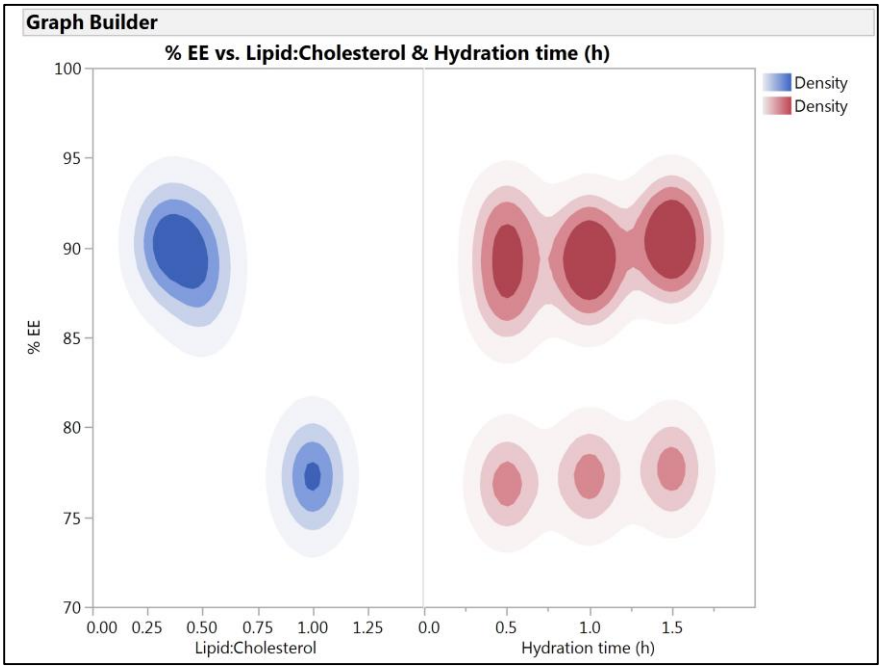

Figure 5.3 Bubble plot showing the effect of independent variables on % EE of RLX-Liposomes (In this figure 0.3 represents 3:1, 0.5 represents 2:1 and 1 represents 1:1 Lipid: Cholesterol ratio on X axis)

Figure 5.3 explains that as the Lipid: Cholesterol ratio is increased, % EE also increases with 3:1 ratio giving high entrapment efficiency. Similarly as the hydration time increased, % EE was also found to increase. However the Response Surface plot, given in Figure 5.4, shows that the Lipid: Cholesterol ratio has more pronounced effect on % EE as compared to the hydration time. Entrapment efficiency is more sensitive towards the factor A than as compared to factor B.

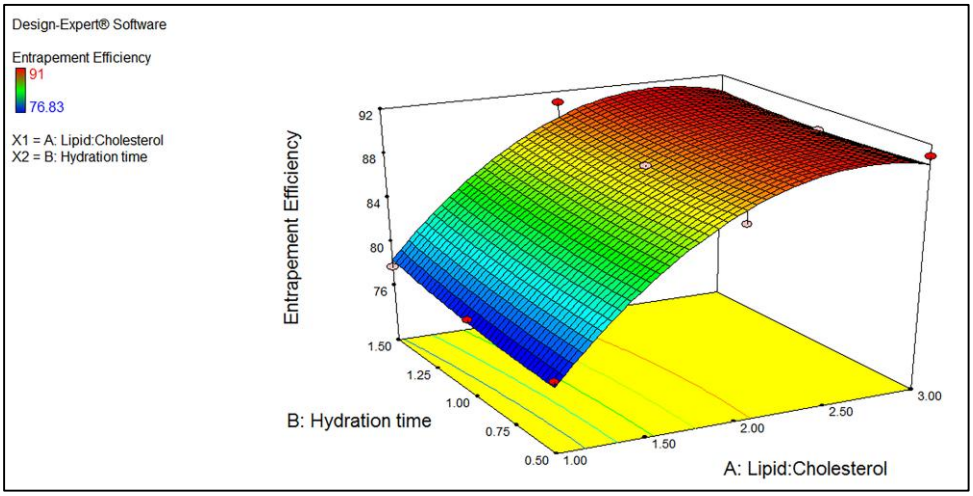

Figure 5.4 Response Surface Plot for showing the effect of independent variables on % EE of RLX-Liposomes

The reason for high entrapment efficiency with increased Lipid: Cholesterol ratio is due to the more concentration of DSPC in the liposomes. DSPC being a high transition temperature lipid forms stable liposomes with less leakage of drugs from the vesicles. The bilayer lipophilic area increases with increasing Lipid: Cholesterol, which accommodates the hydrophobic drug more efficiently. [6]

Hence, based upon these results, desirability criteria obtained using Design Expert software (version 9.0.0.7) was used to find out optimized formulation parameters. Our criteria included maximum % EE and minimum vesicle size. The optimum formulation offered by the software based on desirability was found at 2:1 Lipid: Cholesterol ratio (0 level) with 1.5 hours hydration time (+1 level). The calculated desirability factor for offered formulations was 0.910, which was near to 1 and indicates suitability of the designed factorial model. Figure 5.5 shows the desirability plot.

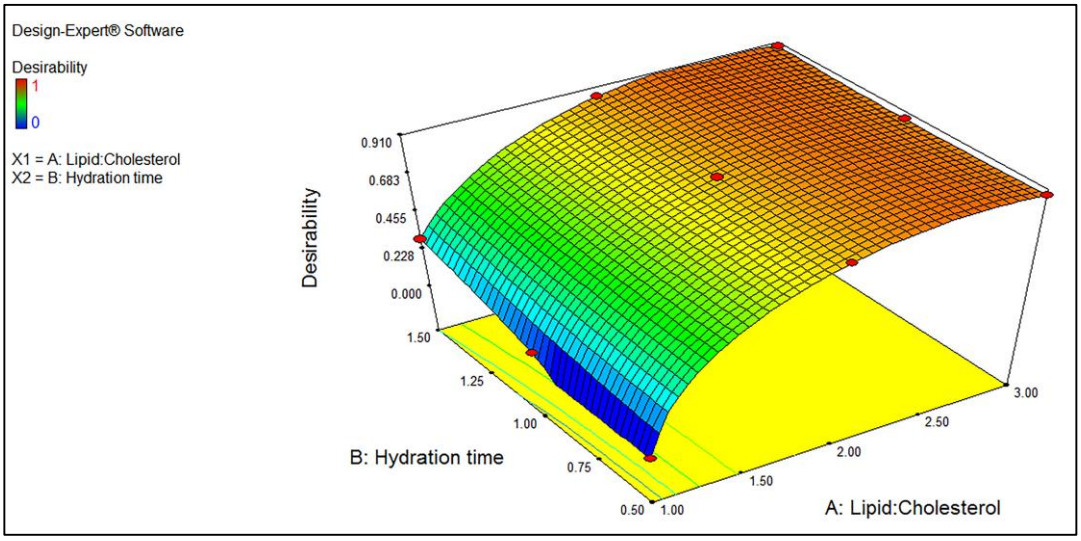

Figure 5.5 Desirability Plot for Optimization of RLX-liposomes

5.3.2.3 Results of Checkpoint Analysis

Two batches were prepared for the checkpoint analysis and results of both vesicle size and % EE (Table 5.3) indicated that the measured response was more accurately predicted by regression analysis that was proven by lower % Error value of regression analysis. Data analysis using t test revealed that there was no statistically significant difference (p value <0.05) between experimentally obtained values and predicted values by regression analysis. The results have been given in Table 5.3

Table 5.3 Results of Checkpoint analysis of RLX loaded liposomes

Response Parameter	**Check Points**	**Predicted Value**	**Observed Value#**	**Residual**	**% Error**
Vesicle size (nm)	Batch 1	128.33	127.89±1.4	0.44	0.3
	Batch 2	130.15	129.78±1.1	0.37	0.28
% EE	Batch 1	91.03	90.85±1.2	0.18	0.19
	Batch 2	90.91	90.98±1.0	0.07	0.01

#Experiment was done in triplicate (n=3)
*Batch 1: (X: 2.7:1 ; Y:1.3 hr)
*Batch 2: (X: 2.6:1 ; Y:1.5 hr)

The p value obtained for vesicle size using t test was 0.786 and for % EE it was 0.597. Data analysis using t test revealed that there was no statistically significant difference (p value <0.05) between experimentally obtained values and predicted values by regression analysis. This indicates that the polynomial equation is validated.

5.4 Preparation and Optimization of Leuprolide acetate loaded Liposomes

5.4.1 Preparation of liposomal formulation and optimization by Design of Experiment

3^2 full factorial design was applied to prepare LA loaded liposomes using DSPC and Cholesterol. Independent variables chosen were Lipid: Cholesterol ratio (X) and Hydration time (Y), while dependent variables were vesicle size (nm) and % Entrapment Efficiency. Table 5.4 shows the independent factors and their levels studied in 3^2 full factorial design.

Table 5.4 Factors and Levels of factors studied in the design

Independent Variables	Low (-1)	Intermediate (0)	High (+)
Lipid: Cholesterol	1:1	2:1	3:1
Hydration time (hours)	0.5	1	1.5

Blank Small Unilamellar Vesicles (SUVs) (i.e. without any drug) were prepared using thin film hydration method using DSPC and Cholesterol. Briefly, Stock solution of DSPC and Cholesterol was prepared in Chloroform: Methanol mixture (9:1). For it, 20 mg each of DSPC and Cholesterol was weighed individually and transferred to small glass bottles/vials. To it 2 ml of Chloroform: Methanol mixture was added and the contents were shaken properly on vortex mixer. DSPC and Cholesterol solutions were taken in Round Bottom Flask (RBF). RBF was then attached to rotary evaporator with temperature maintained at 60^{o}C for 20 minutes at 120 rpm. After the thin lipid film forms, RBF was removed and nitrogen flushing was done to remove traces of organic solvent. Hydration of lipid film was done using 2 ml distilled water at 60^{o}C. The size of liposomes was then reduced by probe sonication 3 cycles of one minute at amplitude of 60 %, 0.6 s (MSE Soniprep, 150 Plus, London, UK).

Kirby and Gregoriadis first established the preparation of Dehydrated-Rehydrated Vesicles (DRV). [7] To the blank SUV (without drug) suspension as prepared above, Leuprolide acetate (3 mg) was added and the dispersion was frozen at -70 ^{o}C for one hour and followed by placing it in freeze dryer overnight with a shelf temperature of -20 ^{o}C. After freeze drying process, lipid cake formed under went controlled rehydration process with 2 ml of double distilled water being added to lipid cake followed by vigorous agitation using vortex mixer till all the lipid cake was re-dispersed and re-hydrated. The suspension was allowed to hydrate at 60 ^{o}C for 30 minutes.

5.4.1.1 Determination of Vesicle size: The Vesicle size and size distribution of formulations were determined by using Dynamic light scattering using Malvern Zetasizer (NanoZS, Malvern Instruments, UK). 50 µL of liposomal dispersion was added to 2 ml distilled water and it was then analyzed for Vesicle Size and size distribution.

5.4.1.2 Determination of % Entrapment Efficiency: Prepared liposomal dispersions were taken in eppendorf tubes and centrifuged (REMI Laboratory Instruments, Mumbai, India) at 20,000 rpm at 4 ^{o}C for 30 minutes. The liposomal pellet settles down while free drug remains in supernatant. The liposomal pellet was air-dried and lysed using methanol. The contents were appropriately diluted and analyzed using UV spectrophotometry at 279 nm. To confirm the mass balance, free drug present in supernatant was also analyzed by UV-Spectrophotometry. 0.1 ml of supernatant was taken and diluted appropriately and absorbance of resultant solution was measured at 279 nm. % EE was calculated using the formula given below:

$$\% \text{ EE } = \frac{\text{Estimated Entrapped drug in Liposomes}}{\text{Total drug added to formulation}} \times 100$$

Experimental batches with the measured responses have been given in Table 5.5.

Table 5.5 3^2 Full Factorial experimental layout with the measured responses

Batch	X (Lipid: Cholesterol)	Y (Hydration time in hours)	Size (nm)*	% EE*
1	1:1	0.5	460±1.3	47.33±1.2
2	1:1	1	367±1.1	48.66±1.5
3	1:1	1.5	515.4±1.1	52.33±1.1
4	2:1	0.5	450.4±2.3	62.33±2.1
5	2:1	1	450.5±2.1	66.8±1.2
6	2:1	1.5	418.4±1.2	72±1.0
7	3:1	0.5	432.5±2.1	74.63±1.1
8	3:1	1	450.4±1.3	72.45±1.4
9	3:1	1.5	357±1.2	72.41±1.2

*Experiment was done in triplicate (n=3)

Statistical analysis of the experimental data and optimization of the formulation was done using Design Expert software (Version 9.0.0.7, State-Ease Inc., Minneapolis, USA) and JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK). Results obtained were statistically analyzed at 5% level of significance and best fitting model was identified. Based on p value obtained conclusion was drawn whether the model terms are significant or non significant. P value less than 0.05 was considered statistically significant. ANOVA was also applied to test the significance of model terms. Model F value and p value were used to conclude the result. Numerical optimization was performed using this software by setting the desired constraints for various variables to obtain the optimized batch with desired responses. Our optimization criteria/constraints included maximum % EE and minimum vesicular size.

With the help of Response Surface Plots (3D plots), and Bubble plots conclusion was drawn regarding the effect of independent variables on dependent variables. Based upon the polynomial equation generated, the quantitative effect of independent factors was studied on the response values. Over all desirability can be obtained from desirability plots of optimization study. The desirability lies between 0 and 1 and it represents the closeness of a response to its ideal value. If both the quality characteristics reach their ideal values, the individual desirability is 1 for both. Consequently, the total desirability is also 1.

5.4.1.3 Checkpoint Analysis

A checkpoint analysis was performed to confirm the utility of the established plots and polynomial equation in the preparation of liposomes. Values of independent variables (X and Y) were taken from solutions suggested by the software and the values of % EE and vesicle size were calculated by substituting the values in the polynomial equation. LA loaded liposomes were prepared experimentally by taking the amounts of the independent variables (X and Y). Each batch was prepared three times and mean values were determined. Difference in the predicted and mean values of experimentally obtained % EE and vesicle size was compared by using statistical 't' test.

5.4.2 Results and Discussion for Formulation and Optimization of LA-Loaded Liposomes

Nine batches of LA loaded liposomes were prepared using 3^2 full factorial design varying two independent factors viz. Lipid: Cholesterol ratio (X) and Hydration time (Y). Vesicle size (nm) and % EE were taken as dependent variables. The results of the same have been shown in Table 5.5. The main effects of X and Y represent the average result of changing one variable at a time from its low to high value. The values of % EE varied from 47.33 to 74.63 % whereas the vesicle size ranged from 357 to 515 nm.

5.4.2.1 Statistical evaluation of results of vesicle size

When the results of vesicle size were analyzed statistically using a 5% level of significance, p value obtained was more than 0.05, indicating the model terms are insignificant. However looking to the Response Surface plot given in Figure 5.6, it can be noticed that as the hydration time increased, there was a slight increase in the vesicle size. It was though not a significant difference as suggested by the statistical p value of 0.645. Similar observation was drawn for Lipid: Cholesterol ratio. There was very negligible increase in the vesicle size with increasing the ratio. Nevertheless, the P value of 0.401 suggests that the change was not significant.

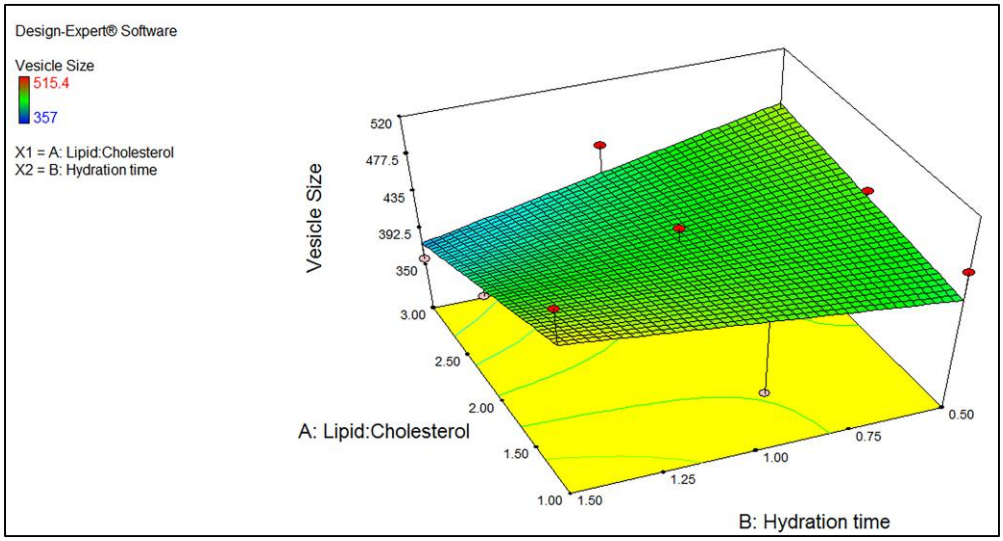

Figure 5.6 Response Surface Plot for showing the effect of independent variables on Vesicle size of LA-Liposomes

The polynomial equation generated for studying the effect of Lipid: Cholesterol and Hydration time was:

Vesicle size= +354.13 + 49.63*A+ 114.8*B -66.4*A*B

Where, A: Lipid: Cholesterol; B: Hydration time.

The reason for increase in vesicle size with increase in hydration time can be attributed to more number of lamellas forming around the aqueous core due to complete rehydration of the dehydrated SUVs. It can be interpreted from the equation as well as Bubble plot as given in Figure 5.7, that vesicle size of dehydrated-rehydrated vesicles in more sensitive towards Hydration time than to the Lipid: Cholesterol ratio.

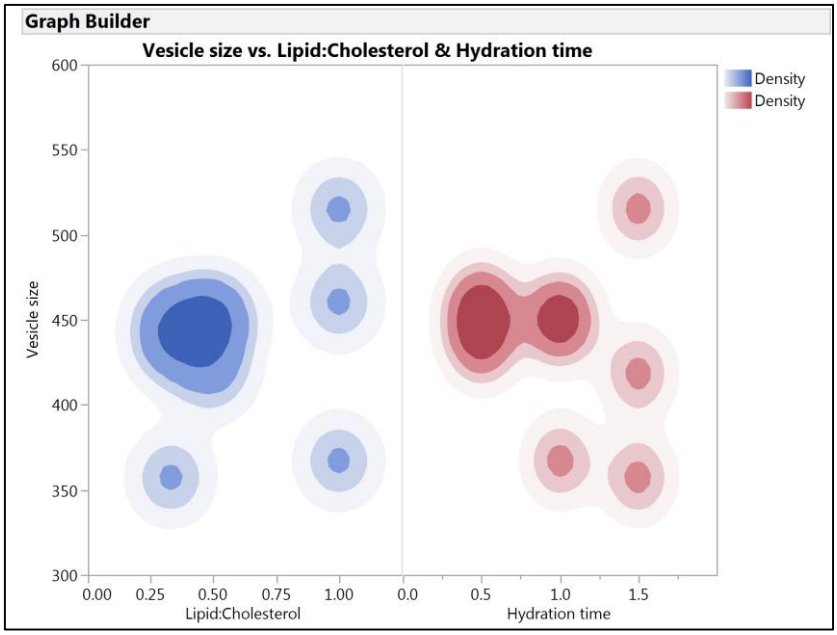

Figure 5.7 Bubble plot showing the effect of independent variables on Vesicle size of LA-Liposomes (In this figure 0.3 represents 3:1, 0.5 represents 2:1 and 1 represents 1:1 Lipid: Cholesterol ratio on X axis)

5.4.2.2 Statistical evaluation of results of % EE

When the results of % EE were analyzed statistically using a 5% level of significance, p value obtained was 0.0001, indicating the model terms are significant. The polynomial equation obtained after regression analysis using the software Design Expert (Version 9.0.0.7, State-Ease Inc., Minneapolis, USA) was:

% EE= +33.85+ 12.63*A+ 4.91*B

where, A is Lipid: Cholesterol; B is Hydration time

Positive sign before the coefficient values of A and B in the equation represents that as the value of that particular factor is increased, the response value increases. Also it can be explained from the value of the coefficients before the factor in the equation that Lipid: Cholesterol has more effect on % EE than Hydration time. The same can the explained from the Response Surface plot given in Figure 5.8.

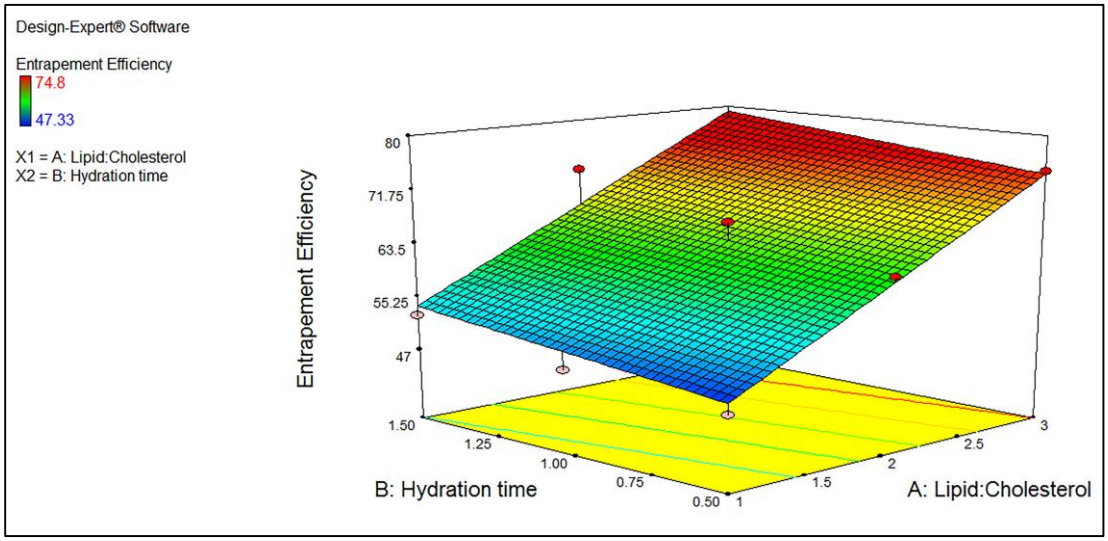

Figure 5.8 Response Surface Plot for showing the effect of independent variables on % EE

Analysis of Variance (ANOVA) was applied for estimation of significance of the model using a 5% significance level. The Model F-value of 42.48 implies that the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case Prob > F was less than 0.0001 for Lipid: Cholesterol ratio while for Hydration time it was 0.1219. This indicates that Lipid: Cholesterol has more significant effect on % EE than the Hydration time. The same can be explained using the bubble plot given in Figure 5.9.

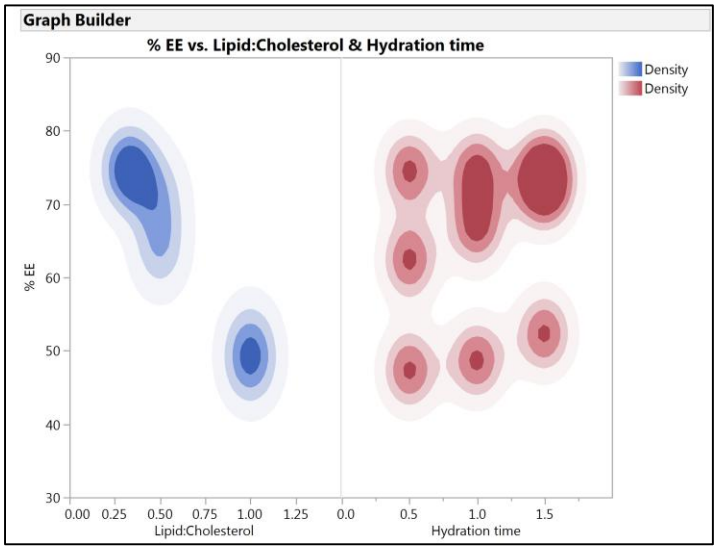

Figure 5.9 Bubble plot showing the effect of independent variables on % EE of LA-Liposomes (In this figure 0.3 represents 3:1, 0.5 represents 2:1 and 1 represents 1:1 Lipid: Cholesterol ratio on X axis)

The liposome surface is proportional to the lipid concentration; in contrast the encapsulated inner volume is proportional to the lipid concentration. Hence increasing the Lipid concentration in liposomes increases the entrapment efficiency. Moreover DSPC has a high transition temperature (56 ^{o}C) due to long alkyl lipid chain and has the strongest chain-chain interactions resulting in closely packed structure. Longer chain alkyl lipids produce more stable liposomes, since the longer alkyl chains, with stronger cohesion, results in less leakage of drug from the aqueous core of liposomes. Thus, it can also provide enhanced encapsulation efficiency for hydrophilic drugs. [6]

To the study the effect of vesicle size on the entrapment efficiency of hydrophilic drug, bubble plot was plotted using JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK). Figure 5.10 explains that with an increase in vesicle size, the % EE also increased. With increase in size, the number of lamellae in the vesicular structures increases, resulting eventually in higher entrapment of hydrophilic drug in the prepared vesicles. [8] Rehydrating the SUVs, results in larger vesicles with more aqueous space to entrap the hydrophilic drug.

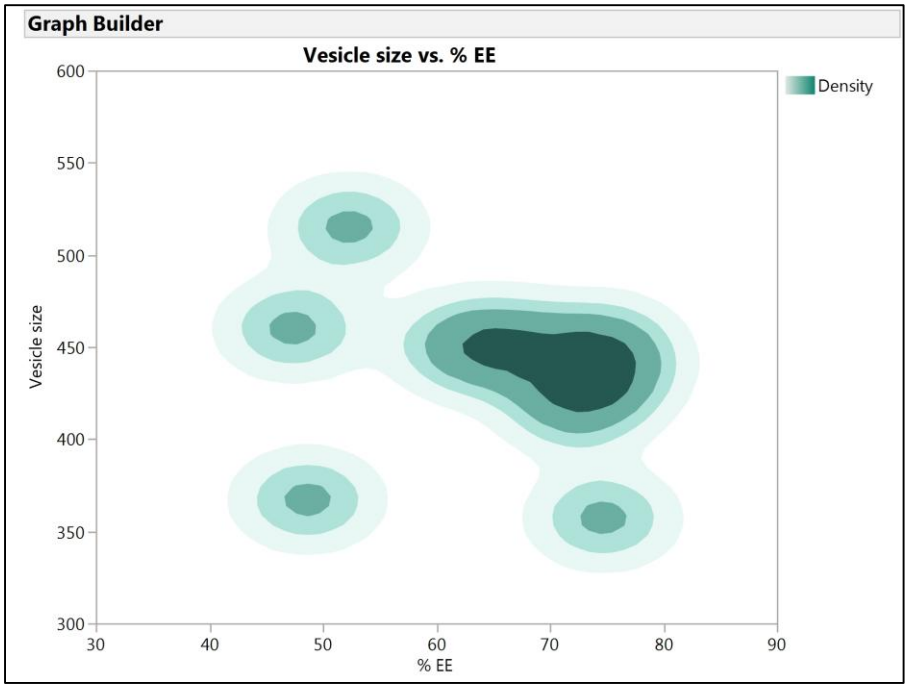

Figure 5.10 Bubble plot to show the relationship between Vesicle size and % EE of LA-Liposomes

Hence, based upon these results, desirability criteria obtained using Design Expert software (version 9.0.0.7) was used to find out optimized formulation parameters. Our criteria included maximum % EE with minimum vesicle size possible. The optimum formulation offered by the software based on desirability was found at 3:1 Lipid: Cholesterol ratio (+1 level) with 0.5 hours hydration time (-1 level). The calculated desirability factor for offered formulations was 0.937, which was near to 1 and indicates suitability of the designed factorial model. Figure 5.11 shows the desirability plot.

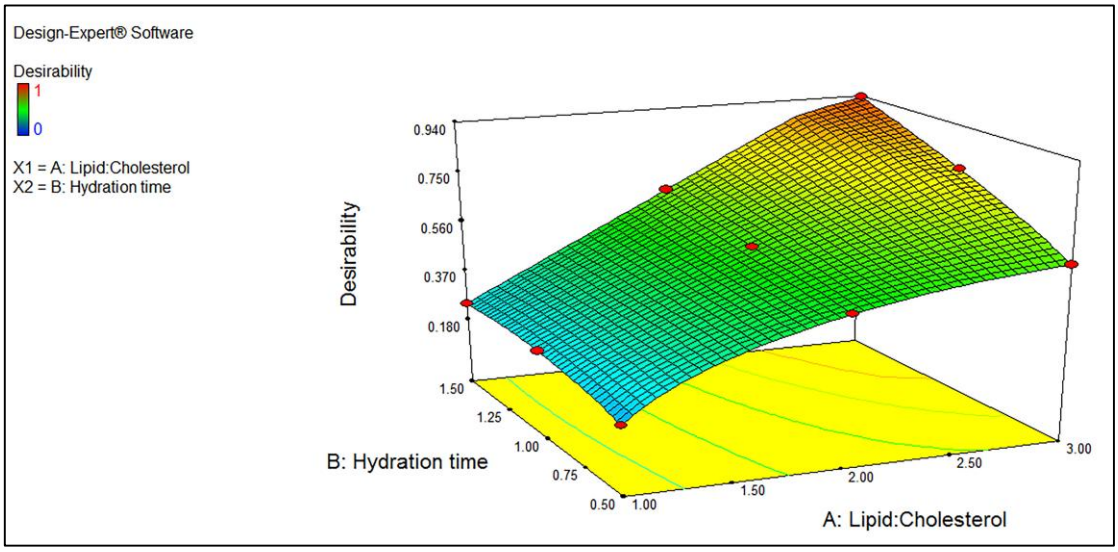

Figure 5.11 Desirability Plot for Optimization of LA-liposomes

5.2.2.3 Results of Checkpoint Analysis

Two batches were prepared for the checkpoint analysis and results of both vesicle size and % EE indicated that the measured response was more accurately predicted by regression analysis that was proven by lower % Error value of regression analysis. Data analysis using t test revealed that there was no statistically significant difference (p value <0.05) between experimentally obtained values and predicted values by regression analysis. The results have been given in Table 5.6.

Table 5.6 Results of Checkpoint analysis of LA loaded liposomes

Response Parameter	Check Points	Predicted Value	Observed Value#	Residual	% Error
Vesicle size (nm)	Batch 1	431.65	430.92±1.1	0.73	0.16
	Batch 2	432.12	431.90±1.2	0.22	0.05
% EE	Batch 1	73.03	72.95±1.1	0.08	0.10
	Batch 2	72.89	72.10±1.0	0.79	1.08

#Experiment was done in triplicate (n=3)
*Batch 1: (X: 2.97: 1; Y:0.33 hr)
*Batch 2: (X: 2.95: 1; Y:0.40 hr)

The p value obtained for vesicle size using t test was 0.503 and for % EE it was 0.489. Data analysis using t test revealed that there was no statistically significant difference (p value <0.05) between experimentally obtained values and predicted values by regression analysis. This indicates that the polynomial equation is validated.

5.5 Preparation and Optimization of dual drug loaded (Raloxifene Hydrochloride and Leuprolide acetate) Liposomes

5.5.1 Preparation of liposomal formulation and optimization by Design of Experiment

3^2 full factorial design was applied to prepare RLX and LA loaded liposomes using DSPC and Cholesterol. Independent variables chosen were Lipid: Cholesterol ratio (X) and Hydration time (Y), while dependent variables were vesicle size (nm) and % Entrapment Efficiency. Table 5.7 shows the independent factors and their levels studied in 3^2 full factorial design.

Table 5.7 Factors and Levels of factors studied in the design

Independent Variables	Low (-1)	Intermediate (0)	High (+)
Lipid: Cholesterol	1:1	2:1	3:1
Hydration time (hours)	0.5	1	1.5

To prepare the dual drug loaded liposomes, DSPC, Cholesterol and Raloxifene Hydrochloride (6 mg) were taken in Round Bottom Flask (RBF). RBF was then attached to rotary evaporator with temperature maintained at 60 ^{o}C for 20 minutes at 120 rpm. After the thin lipid film forms, RBF was removed and nitrogen flushing was done to remove traces of organic solvent. Hydration of lipid film was done using 2 ml distilled water at 60 ^{o}C. The size of liposomes was then reduced by probe sonication 3 cycles of one minute at amplitude of 60 %, 0.6 s (MSE Soniprep, 150 Plus, London, UK). To the liposomal dispersion loaded with Raloxifene Hydrochloride, Leuprolide acetate (3mg) was added. This dispersion was then frozen at -70 ^{o}C for one hour and then placed in freeze dryer overnight with a shelf temperature of -20 ^{o}C. After freeze drying process, lipid cake formed under went rehydration process with 2 ml of double distilled water being added to lipid cake followed by vigorous agitation using vortex mixer till all the lipid cake was re-dispersed and re-hydrated. The suspension was allowed to hydrate at 60 ^{o}C for 30 minutes.

5.5.1.1 Determination of Vesicle size: The Vesicle size and size distribution of formulations were determined by using Dynamic light scattering using Malvern Zetasizer (NanoZS, Malvern Instruments, UK). 50 µL of liposomal dispersion was added to 2 ml distilled water and it was then analyzed for Vesicle Size and size distribution.

5.5.1.2 Determination of % Entrapment Efficiency: Prepared liposomal dispersions were taken in eppendorf tubes and centrifuged (REMI Laboratory Instruments, Mumbai, India) at 20,000 rpm at 4 ^{o}C for 30 minutes. The liposomal pellet settles down while free drug remains in supernatant. The liposomal pellet was air-dried and lysed using methanol. The contents were appropriately diluted and analyzed using UV spectrophotometry at 279 nm and 287 nm to estimate the concentrations of LA and RLX respectively. To confirm the mass balance, free drug present in supernatant was also analyzed by UV-Spectrophotometry. 0.1 ml of supernatant was taken and diluted appropriately and absorbance of resultant solution was measured at 279 nm and 287 nm for LA and RLX respectively. % EE was calculated using the formula given below:

$$\% \text{ EE } = \frac{\text{Estimated Entrapped drug in Liposomes}}{\text{Total drug added to formulation}} \times 100$$

Experimental batches with the measured responses have been given in Table 5.8.

Table 5.8 3^2 Full Factorial experimental layout with the measured responses

Batch	X	Y	% EE*		Size (nm)*
			RLX	LA	
1	1:1	0.5	76.71±1.2	46.66±1.4	211.6±1.1
2	1:1	1	77.1±1.1	48.33±2.1	228.7±2.2
3	1:1	1.5	77.5±1.1	51.6±1.5	261.7±1.5
4	2:1	0.5	87.33±1.0	61.6±1.7	290.2±2.1
5	2:1	1	88.4±1.2	66.3±1.8	310.1±1.4
6	2:1	1.5	89.8±1.3	71.2±1.4	352.9±2.1
7	3:1	0.5	90.91±1.1	74.3±1.0	354.4±1.0
8	3:1	1	89.6±1.4	72.6±1.2	388.8±1.3
9	3:1	1.5	89.4±1.3	72.3±1.1	398.3±1.1

*Experiment was done in triplicate (n=3)

Statistical analysis of the experimental data and optimization of the formulation was done using Design Expert software (Version 9.0.0.7, State-Ease Inc., Minneapolis, USA) and JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK). Results obtained were statistically analyzed at 5% level of significance and best fitting model was identified. Based on p value obtained conclusion was drawn whether the model terms are significant or non significant. P value less than 0.05 was considered statistically significant. ANOVA was also applied to test the significance of model terms. Model F value and p value were used to conclude the result. Numerical optimization was performed using this software by setting the desired constraints for various variables to obtain the optimized batch with desired responses. Our optimization criteria/constraints included maximum % EE and minimum vesicular size.

With the help of Response Surface Plots (3D plots), and Bubble plots conclusion was drawn regarding the effect of independent variables on dependent variables. Based upon the polynomial equation generated, the quantitative effect of independent factors was studied on the response values. Over all desirability can be obtained from desirability plots of optimization study. The desirability lies between 0 and 1 and it represents the closeness of a response to its ideal value. If both the quality characteristics reach their

ideal values, the individual desirability is 1 for both. Consequently, the total desirability is also 1.

5.5.1.3 Checkpoint Analysis

A checkpoint analysis was performed to confirm the utility of the established plots and polynomial equation in the preparation of liposomes. Values of independent variables (X and Y) were taken from solutions suggested by the software and the values of % EE and vesicle size were calculated by substituting the values in the polynomial equation. RLX-LA loaded liposomes were prepared experimentally by taking the amounts of the independent variables (X and Y). Each batch was prepared three times and mean values were determined. Difference in the predicted and mean values of experimentally obtained % EE and vesicle size was compared by using statistical 't' test.

5.5.2 Results and Discussion for Formulation and Optimization of dual drugs RLX and LA Loaded Liposomes

Nine batches of RLX-LA loaded liposomes were prepared using 3^2 full factorial design varying two independent factors viz. Lipid: Cholesterol ratio (X) and Hydration time (Y). Vesicle size (nm) and % EE were taken as dependent variables. The results of the same have been shown in Table 5.8. The main effects of X and Y represent the average result of changing one variable at a time from its low to high value.

5.5.2.1 Statistical evaluation of results of vesicle size

When the results of vesicle size were analyzed statistically using a 5% level of significance, p value obtained was less than 0.05, indicating the model terms are significant. P value for Lipid: Cholesterol was 0.0001 and for Hydration time it was 0.0002. This means that any variation in the Lipid: Cholesterol ratio and hydration time will affect the vesicle size significantly. This can be explained by the Response Surface plot given in Figure 5.12. As the Lipid: Cholesterol ratio was increased, vesicle size was found to increase. There was a wide variation in the vesicle size from 211 nm to 398 nm, as seen from the data given in Table 5.8, with increasing the Lipid: Cholesterol ratio and

Hydration time. The reason can be attributed to increase in the total lipid concentration adding more lamellas to the structure with the increasing hydration time.

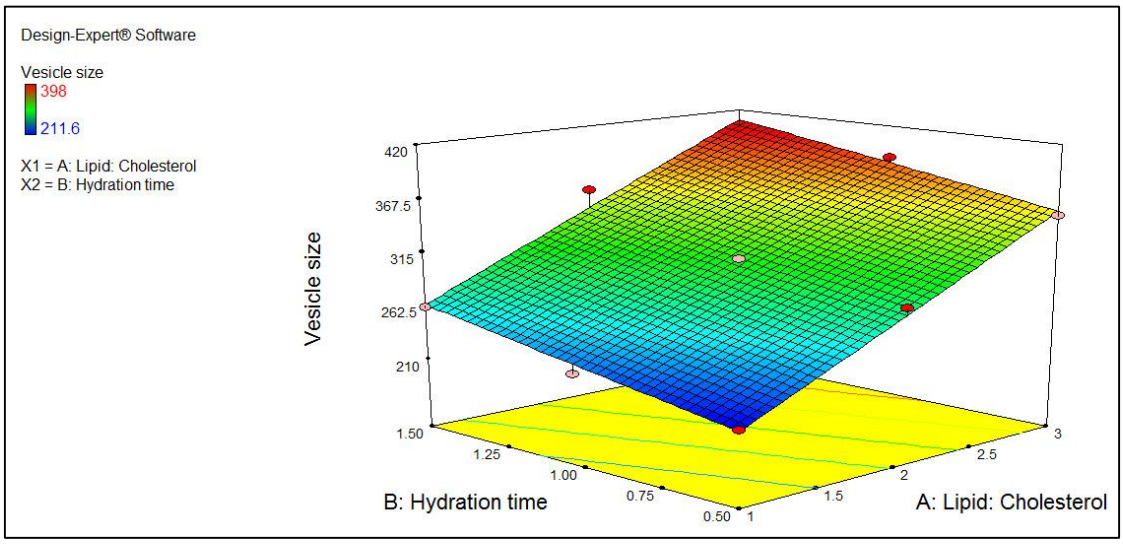

Figure 5.12 Response Surface Plot for showing the effect of independent variables on Vesicle size of dual drug loaded liposomes

The polynomial equation generated for studying the effect of Lipid: Cholesterol and Hydration time on the vesicle size of dual drug entrapped liposomes was:

Vesicle size= +111.00 + 72.91*A+ 53.66*B

Where, A: Lipid: Cholesterol; B: Hydration time.

The reason for increase in vesicle size with increase in hydration time can be attributed to more number of lamellas forming around the aqueous core due to complete rehydration of the dehydrated SUVs. It can be interpreted from the equation as well as Bubble plot as given in Figure 5.13, that vesicle size of dehydrated-rehydrated vesicles in more sensitive towards Lipid: Cholesterol ratio than Hydration time. The co-efficient value of A is more than B and both positively affect the vesicle size.

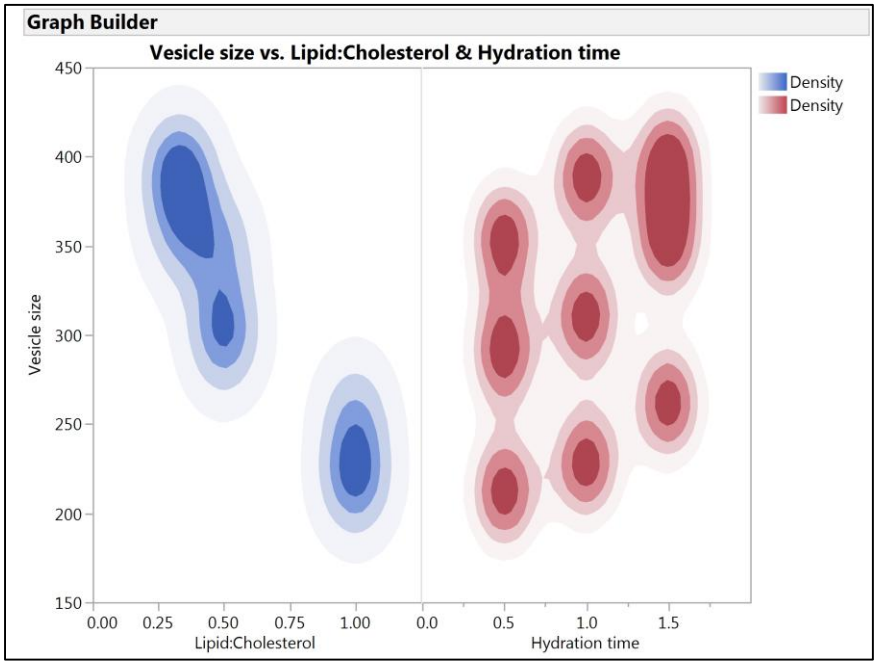

Figure 5.13 Bubble plot showing the effect of independent variables on vesicle size of dual drug loaded liposomes (In this figure 0.3 represents 3:1, 0.5 represents 2:1 and 1 represents 1:1 Lipid: Cholesterol ratio on X axis)

5.5.2.2 Statistical evaluation of results of % EE

When the results of % EE were analyzed statistically using a 5% level of significance, p value obtained was 0.0002, indicating the model terms are significant. The polynomial equation obtained after regression analysis using the software Design Expert (Version 9.0.0.7, State-Ease Inc., Minneapolis, USA) was:

% EE= +54.46+ 25.84*A+ 2.35*B

where, A is Lipid: Cholesterol; B is Hydration time

Positive sign before the coefficient values of A and B in the equation represents that as the value of that particular factor is increased, the response value increases. As the Lipid: Cholesterol ratio is increased, % EE of both the drugs increases due increasing DSPC concentration in the liposomes leading to formation of stable liposomes with larger bilayer structures entrapping the hydrophobic drug RLX while at the same time preventing the leakage of hydrophilic drug LA from its aqueous core. Response Surface plots are given in Figures 5.14 and 5.15 for RLX and LA respectively.

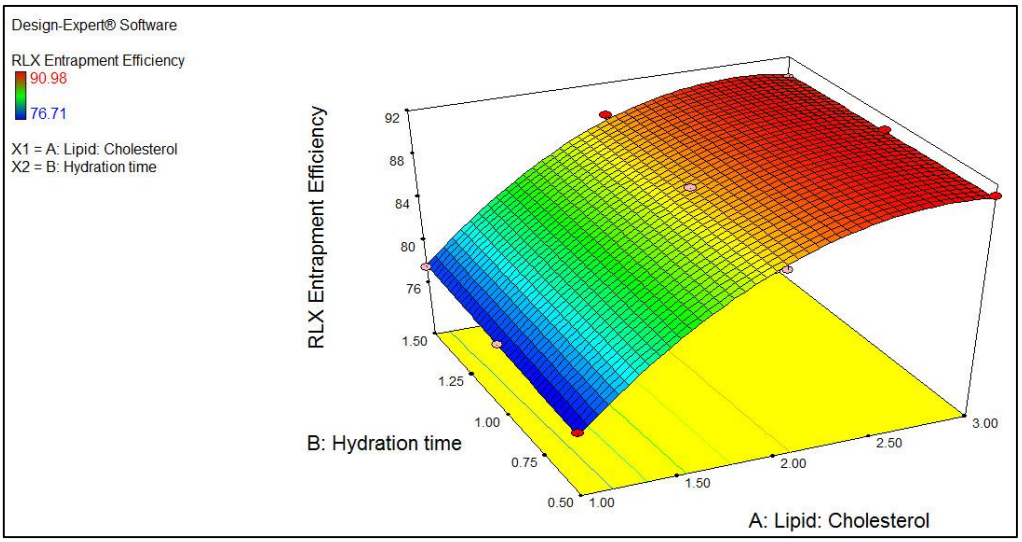

Figure 5.14 Response Surface Plot for showing the effect of independent variables on % EE of RLX in dual drug loaded liposomes

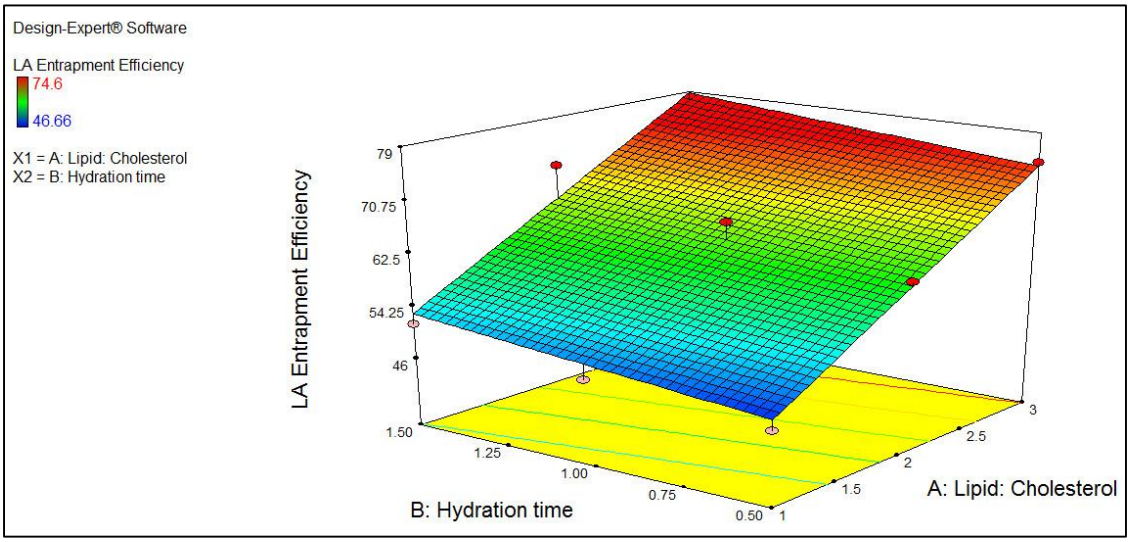

Figure 5.15 Response Surface Plot for showing the effect of independent variables on % EE of LA in dual drug loaded liposomes

Analysis of Variance (ANOVA) was applied for estimation of significance of the model using a 5% significance level. The Model F-value of 414.04 implies that the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. However, p value of Lipid: Cholesterol ratio was 0.0001 while that of Hydration time it was 0.277, which indicate that Lipid: Cholesterol significantly affect the % EE of dual

drugs while the Hydration time has non-significant effect. It can also be explained from the value of the coefficients before the factor in the equation that Lipid: Cholesterol has more significant effect on % EE than Hydration time. Bubble plots were obtained using JMP 12 statistical discovery software (Version 12.2.0, SAS Institute Inc.,UK). Figures 5.16 and 5.17 represents the plot for RLX and LA respectively. Figures show the significant effect of Lipid: Cholesterol than as compared to Hydration time on % EE.

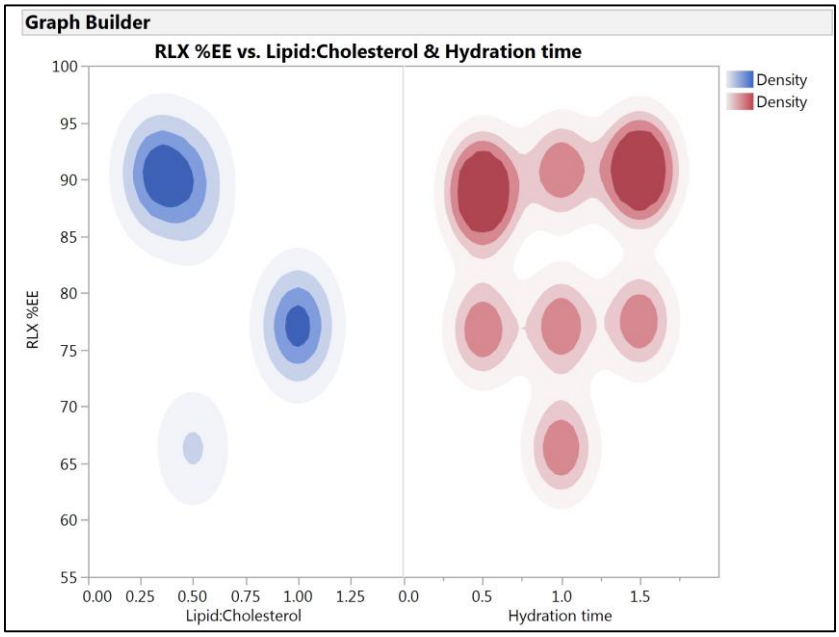

Figure 5.16 Bubble plot showing the effect of independent variables on % EE of RLX in dual drugs loaded liposomes (In this figure 0.3 represents 3:1, 0.5 represents 2:1 and 1 represents 1:1 Lipid: Cholesterol ratio on X axis)

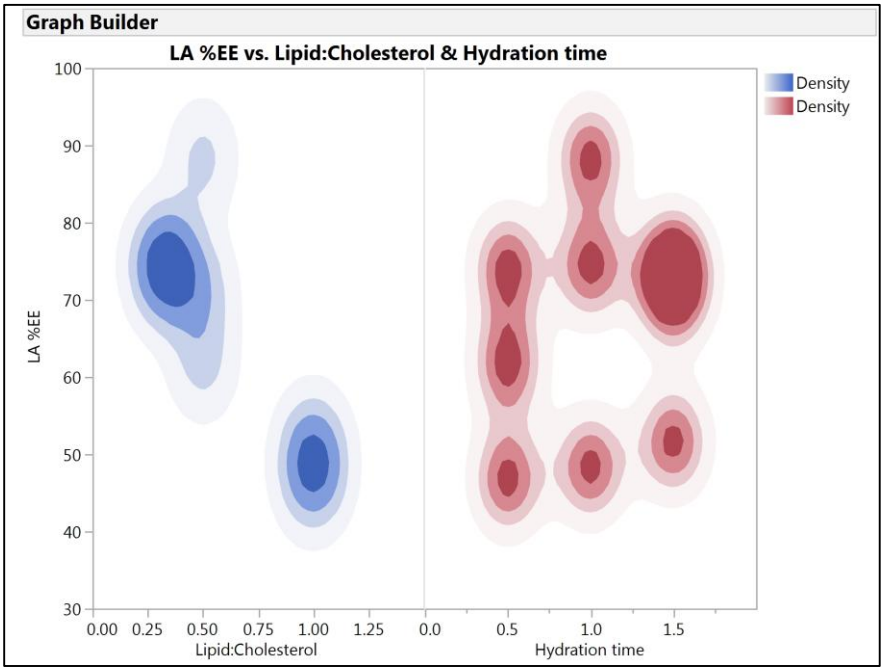

Figure 5.17 Bubble plot showing the effect of independent variables on % EE of LA in dual drugs loaded liposomes (In this figure 0.3 represents 3:1, 0.5 represents 2:1 and 1 represents 1:1 Lipid: Cholesterol ratio on X axis)

To the study the effect of vesicle size on the entrapment efficiency of dual drugs within same liposomal structure, bubble plot was plotted. Figure 5.18 explains that with an increase in vesicle size, the % EE of both the drugs increased. With increase in size, the number of lamellae in the vesicular structures increases, resulting eventually in higher entrapment of both hydrophilic and lipophilic drugs in the prepared vesicles. [8] Rehydrating the SUVs, results in larger vesicles with more aqueous space to entrap the hydrophilic drug while the lipidic bilayers are able to entrap the lipophilic drug.

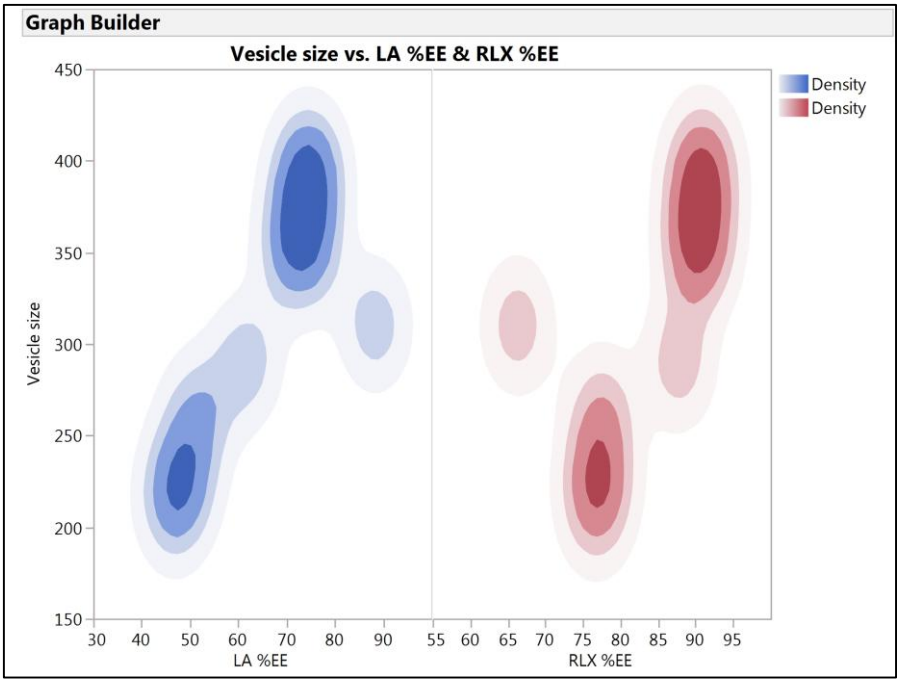

Figure 5.18 Bubble plot to show the relationship between Vesicle size and % EE of LA and RLX in dual drug entrapped liposomes

A bubble plot was also plotted to study the relationship between the % EE of LA and % EE of RLX. Figure 5.19 represents that % EE of both the drugs increased with respect to each other. There was no negative impact of dual drug entrapment.

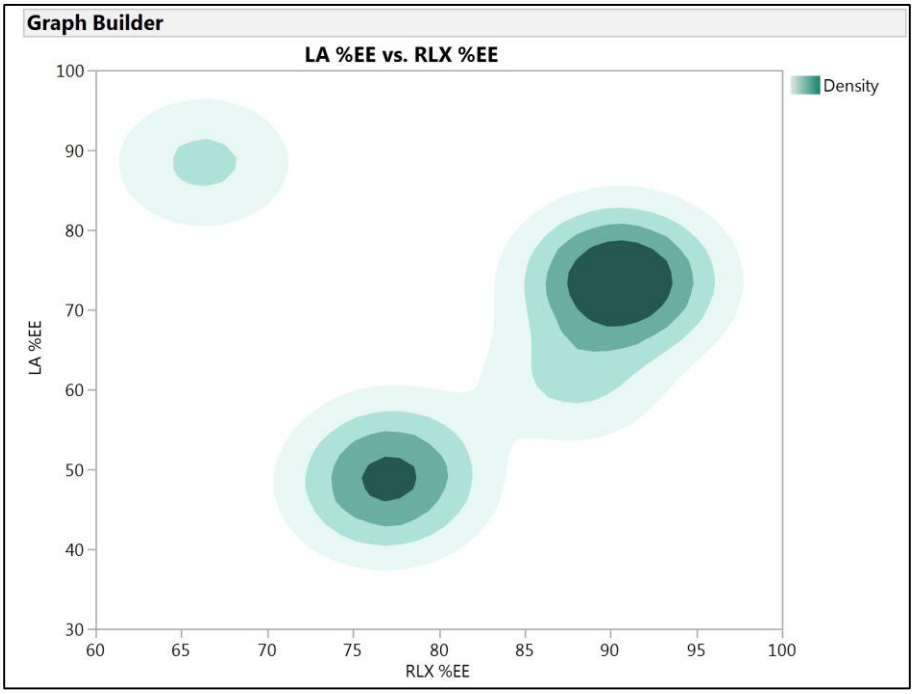

Figure 5.19 Bubble plot to show the relationship between % EE of LA and % EE of RLX in dual drug entrapped liposomes

Hence, based upon these results, desirability criteria obtained using Design Expert software (version 9.0.0.7) was used to find out optimized formulation parameters. Our criteria included maximum % EE with minimum vesicle size possible. The optimum formulation offered by the software based on desirability was found at 3:1 Lipid: Cholesterol ratio (+1 level) with 0.5 hours hydration time (-1 level). The calculated desirability factor for offered formulations was 0.673, which indicates suitability of the designed factorial model. Figure 5.20 shows the desirability plot.

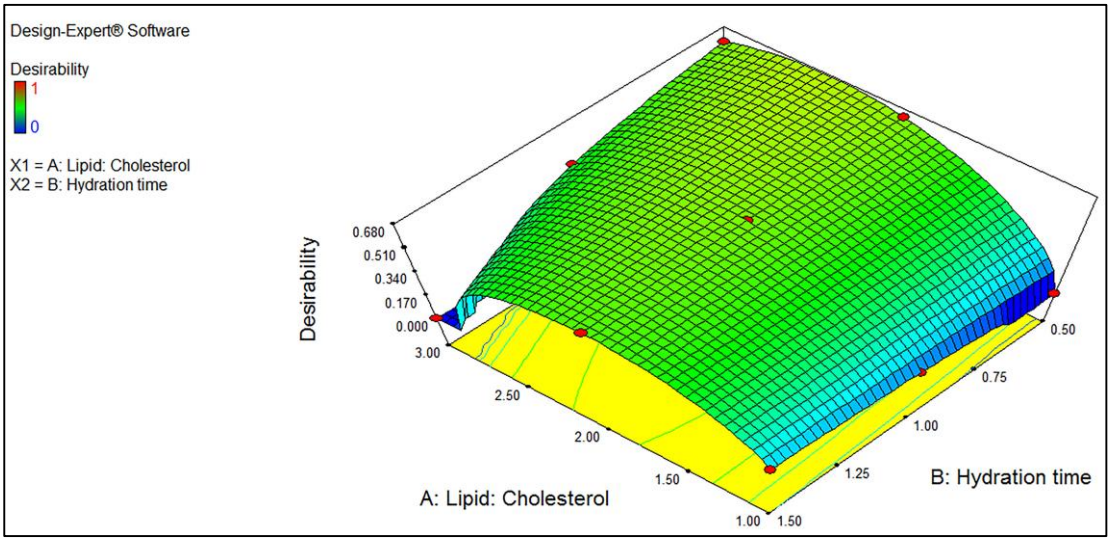

Figure 5.20 Desirability Plot for Optimization of Dual drug (RLX and LA) loaded liposomes

5.5.2.3 Results of Checkpoint Analysis

Two batches were prepared for the checkpoint analysis and results of both vesicle size and % EE indicated that the measured response was more accurately predicted by regression analysis that was proven by lower % Error value of regression analysis. Data analysis using t test revealed that there was no statistically significant difference (p value <0.05) between experimentally obtained values and predicted values by regression analysis. The results have been given in Table 5.9.

Table 5.9 Results of Checkpoint analysis of Dual drug loaded liposomes

Response Parameter	**Check Points**	**Predicted Value**	**Observed Value#**	**Residual**	**% Error**
Vesicle size (nm)	Batch 1	320.96	319.75±1.3	1.21	0.37
	Batch 2	312.26	311.12±1.2	1.14	0.36
% EE	Batch 1	RLX-90.69	RLX-90.98±1.2	0.29	0.31
		LA-67.57	LA-67.85±1.2	0.28	0.41
	Batch 2	RLX-90.32	RLX-89.95±1.1	0.4	0.40
		LA-66.05	LA-66.20±1.3	0.15	0.22

#Experiment was done in triplicate (n=3)
*Batch 1: (X: 2.5: 1; Y:0.5 hr)
*Batch 2: (X: 2.4: 1; Y:0.5 hr)

The p value obtained for vesicle size using t test was 0.86, which indicates that the difference is not significant at $p<0.05$. Similarly for % EE, p value for RLX was 0.95 and for LA it was 0.85 indicating that the difference is not significant at $p<0.05$. This shows that the polynomial equation is validated.

5.6 Preparation of liposomal formulations loaded Intravaginal Rod Insert

The intravaginal inserts, in the form of lyophilised liposomal gels swell in contact with vaginal fluid and release the formulation for targeting to uterus through First Uterine Pass Effect. It comprises of an elastomeric body made up of medical grade silicone, which holds the liposomal formulation as freeze -dried rod. Intravaginal rods were prepared from all the three optimized batches of liposomes: RLX loaded liposomes, LA- loaded liposomes and dual drug (RLX and LA) loaded liposomes. The method of preparation of liposomes loaded IVRs were same for all the three formulations.

To the 2 ml of liposomal dispersion, 450mg of mannitol (Sigma Aldrich, USA) and 35mg of gelatin (Sigma Aldrich, USA) was added to obtain a gel like consistency. The gel was mixed at 3000 rpm for 9 minutes at 25 ^{o}C in a centrifuge (Remi Equipment, Mumbai, India). This was then inserted into silicone tubing (2mm int. diameter, 4mm ext. diameter, VWR International, UK) using a syringe. The tubing was then frozen at -80 ^{o}C for 4 hours. This was then cut into 1 cm sections and the rods were kept for freeze-drying using programmable freeze dryer (VirTis Advantage, SP Scientific, UK). The sections were warmed to -50 ^{o}C (held for 90 min) followed by drying at 200 mtorr (-50 ^{o}C, 360 min; -40 ^{o}C, 120 min ramp and 120 min hold, -30 ^{o}C, 120 min ramp and 180 min hold) followed by drying at 585 mtorr (-20 ^{o}C, 120 min) and 600 mtorr (-20 ^{o}C, 840 min ramp, -10 ^{o}C, 600 min hold, 0 ^{o}C, 60 min ramp, 120 ramp, 20 ^{o}C, 60 min ramp and finally held at 600m torr, 30 ^{o}C for 960 min. The liposomal dispersion gets freeze-dried as rods in the silicone tubing. [9, 10]

These liposomal formulations loaded Intravaginal Rods were then further characterized for in vitro drug release and stability studies and were used to the study the efficacy of the developed formulations in uterine fibroid induced rabbit model.

5.7 References

1) Sangshetti, J.N., Deshpande, M., Zaheer, Z., Shinde, D.B. and Arote, R., 2014. Quality by design approach: Regulatory need. Arabian Journal of Chemistry. http://dx.doi.org/10.1016/j.arabjc.2014.01.025.

2) Gawade, A., Chemate, S. and Kuchekar, A., 2013. Pharmaceutical Quality by Design: A new approach in product development. Res. Rev.: J. Pharm. Pharm. Sci, 2(3), pp.5-12.

3) Miller AJ. 1984. Selection of subsets of regression variables. J. R. Stat. Soc. Ser. A. 147:389-425.

4) Wagner JM, Shimshak DG. 2007. Stepwise selection of variables in data envelopment analysis: Procedures and managerial perspectives. Eur. J. Oper. Res. 180(1): 57- 67.

5) Myer RH, Montogomery DC. 2002. Response Surface Methodology: Process and product optimization using designed experiment. New York: John Wiley and Sons.

6) Dan, 2007. Lipid tail chain asymmetry and the strength of membrane-induced interactions between membrane proteins Biochim. Biophys. Acta; 1768: 2393–2399.

7) Kirby, C. and Gregoriadis, G., 1984. Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. Nature Biotechnology, 2 (11), pp.979-984.

8) Jain B, Singh B, Katare OP, and Vyas SP 2010. Development and characterization of minoxidil-loaded liposomal system for delivery to pilosebaceous units. Journal of Liposome Research; 20(2): 105–114.

9) Pattani, A, Lowry, D, Curran, RM, McGrath, S, Kett, VL, Andrews, GP & Malcolm, RK 2012. Characterisation of protein stability in rod-insert vaginal rings. International Journal of Pharmaceutics; 430 (1-2): 89-97.

10) Gupta, P.N., Pattani, A., Curran, R.M., Kett, V.L., Andrews, G.P., Morrow, R.J., Woolfson, A.D. and Malcolm, R.K., 2012. Development of liposome gel based formulations for intravaginal delivery of the recombinant HIV-1 envelope protein CN54gp140. European Journal of Pharmaceutical Sciences, 46(5), pp.315-322.