Lipoplex Delivery System for P11 Gene: A Risk Based Quality by Design Approach

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Abstract

The purpose of this study was to explore Quality by Design (QbD) principle for the preparation of lipoplex containing p11 cDNA to prove both feasibility and convenience of executing QbD concept to liposome based gene delivery system. Product and process indulgence will assist in decreasing the inconsistency of critical material and process parameters, which provide quality in productivity and diminish the risk. This study encompasses identification of the Quality Target Product Profiles (QTPPs) and Critical Quality Attributes (CQAs) from preliminary studies. To classify and control the unpredictability in process and material attributes, two implements of QbD was exploited, Quality Risk Management (QRM) and Experimental Design. Additionally, it aids to ascertain the outcome of these attributes on CQAs. Potential risk factors were recognised from fishbone diagram and screened by risk assessment and optimized by D-optimal experimental design, to analyse the precision of the target process. This optimized formulation was further characterized by for their size, PDI and complexation efficiency. Design space was created using experimental design tool that gives the control space and working within this controlled space reduces all the failure modes below the risk level. In conclusion, QbD approach with QRM tool provides potent and effectual pyramid to enhance the quality into lipoplex.

Introduction

Gene delivery is a development such that foreign DNA is transported to host cells for uses such as genetic research or gene therapy to cure disease with its root cause. For the last few years, many efforts have been made to construct a vector which is not originated from virus and results in discovery of non-viral vectors allowing a superior flexibility in the capacity of DNA transportation, bypassing the immunity of host body and increase the safety. Because of their positive charge, cationic lipids, unsurprisingly form complex with the negatively charged DNA. In this way, plasmid DNA can be covered with lipids in an organized structure like a micelle or a liposome. This organised structure with DNA is called lipoplex which is fundamentally, a liposome complexed DNA. The structural similarity of liposome bilayers to cellular membranes has intrigued scientists to explore the potential using liposomes as drug and DNA to deliver therapeutics with different properties to specific regions of the body since the early 1970s. Typically lipoplex carry the net positive charge value that allow the complex to interact with the cell membrane containing negative charge and results into internalisation by the cell which occur through any process of endocytosis. Furthermore, fusogenic lipids present in lipoplex formulation help in the endosomal escape resulting in increased transfection efficiency of the formulation [1]. Besides, during the process of gene delivery, there are a series of hindrances to circumnavigate. However, development of liposomal complexes using lipids with varied properties navigate the multiple obstacles is also desired [2].

The structural versatility of liposomes allows the incorporation of lipid-soluble, water-soluble drugs, proteins, peptides and even DNA molecules in the bilayers and the aqueous compartment, respectively [3]. Compared to hydrophobic molecules, the encapsulation of hydrophilic molecules like DNA in lipoplex presents unique challenges. In addition, it is unknown how different process and product variables impact on product quality and performance. There are several factors that may have
contributed to the slow paced research of lipoplex containing DNA: 1) The difficulties associated with identifying the formulation and process design critical quality attributes of these complex systems; 2) The high manufacturing cost due to low reproducibility and low entrapment of therapeutic active agents; 3) The high regulatory burden associated with product safety of these complex parenteral bioprodut.

Therefore, investigation of the application of Quality by Design (QbD) concepts to liposomes containing a therapeutic gene, p11 cDNA was used as a model compound. The p11 gene assists in regulating the signalling of serotonin, a neurotransmitter targeted by many antidepressants and tangled to mood, sleep and memory. The implementation of QbD methodologies to the development of liposome containing p11 cDNA requires the definition of a Quality Target Product Profile (QTPP) as a foundation for presentation, and the empathy of those quality features that are critical (CQA) and necessity to control sensibly to keep product reliability and usefulness. Nevertheless, existing QbD execution, as clear by current ICH Q8 (R2) and succeeding guidelines is principally restricted to considerate manufacturing process, but does not incorporate aspects of product knowledge, such as product design and product stipulations for proposed routine [4]. In this framework, “developability” can, in fact, be measured as an allowance of QbD guidance, providing a bond amid “product understanding and method understanding” and serving to develop the design space for a proper drug candidate. Developability can be useful for initial derisking and in what way it can be flawlessly united equally with detection and progression of development goings-on.

Even before a central contender is established, such necessities can be briefed in an envisioned performance outline, and from that outline one can develop the obligatory features that will aid in ensuring the development of a high quality therapeutic candidate. In this background, developability addresses more than just “purityness” or “steadiness” of the manufactured product. It also offers a platform to include a solid foundation for “product knowledge” and describes, right from the beginning, a robust QTPP, an implement of QbD, that would significantly upsurge the probabilities of an effective, harmless, and effective product [5]. In short, the developability profile of a given new drug candidate is sustained by three “quality areas” or “pillars” that ultimately define its performance i.e. manufacturability, safety and its pharmacology that is highly dependent on the size and size distribution of lipoplex i.e. a Nanosized career. Risk assessment, another tool of QbD, consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards. It targets at giving responses to the subsequent three questions in the study, 1) What might be mistaken? 2) What is the probability of that mistake? 3) What are the magnitudes (severity) of that mistake? The assessment of the threat to quality should be built on technical acquaintance and eventually bond to the fortification of the enduring, the level of exertion and convention.

In the present study, various liposomal formulations containing p11 cDNA were prepared in a structured manner, an organized method for determining the relationship between factors affecting a process and the output of that process is known as “Design of Experiments” (DoE). A commendable design is set up on comprehensive understanding of product and genuine observation of entire progression during manufacturing. DoE studies work in organisation with mechanism based studies to attain improved product and process considerate. With respect to the preparation process, the impact of various process parameters on the liposome particle size, Polydispersity Index (PDI), as well as DNA complexation efficiency was analysed.

**Experimental Methods**

**Preparation of cationic liposomes**

The lipid stock solutions were obtained by dissolving the all lipids (HSPC, DPPE, Cholesterol, PEG 2000-DSPE and DOPE) along with fixed quantity of DOPE and positively charged DOTAP, in appropriate quantities in a mixture of chloroform and methanol (3:1 v/v) mixture in a 50 mL RBF. The solvents methanol and chloroform
were then evaporated by using a vacuum rotary evaporator system (IKA RV-10, USA) at -400 to -500 mm HG vacuum and 45-50 °C temperature, at 120 RPM to form a thin lipid film on the wall of RBF. Thin film thus formed was hydrated with DNase free water-DFW at 65 °C temperature and subsequently, formed liposomes were extruded through each of 1 µ, 0.8 µ, 0.4 µ, 0.2 µ, and 0.1 µ polycarbonate membranes (Whatman, USA) supported by polyethylene drain disk (Whatman, USA) using high pressure extruder (Avestin, USA) for 5-7 cycles [6].

**Determination of size and PDI (polydispersity index)**

Appropriately diluted fixed volume of liposomal formulation was taken in clear disposable sample cuvettes and measurements were performed in triplicate using Malvern Zeta sizer Nano ZS (Malvern Instruments Inc., UK).

**Preparation of lipoplex**

Process parameters involved in the complexation of cDNA with cationic liposomes were also optimized. The cDNA was added to the diluted cationic liposomes and the samples were vortexed and maintained at room temperature for 30 minutes to promote the cDNA association with the cationic liposomes [7]. Different L/P ratios (ratio of moles of cationic lipid to moles of phosphates of cDNA) were prepared by adding increasing concentrations of 5 µL cDNA solution to 20 µL of cationic liposomes to optimise the maximum complexation efficiency of liposomes.

**EtBr intercalation assay using gel electrophoresis**

Lipoplexes prepared by incubating cDNA with cationic liposomes were subjected for determination of complexation efficiency of cationic liposomes with negatively charged cDNA [8]. Prepared lipoplexes, diluted if necessary, were loaded on to gel at level of 200 ng/well using gel loading buffer and electrophoresis was carried out at 5 V/cm for 45 min in 1X TAE buffer. Uncomplexed cDNA migrated on gel was visualized by UV transillumination on Gel Doc Image XR + system (Bio-Rad Labs., USA). Amount of free cDNA was quantified using naked DNA band as reference and determining the band densities using ImageJ software ver. 1.50 c (National Institute of Health, USA).

**Optimization of process parameters**

Diverse process parameters concerned in the preparation of lipoplexes were optimized primarily. These process parameters comprised of incubation temperature, incubation time and pH. Retardation of comprehensive quantity of cDNA on gel by lipoplexes was considered as optimum process parameter. While retaining one factor persistent, outcome of alternative variables was observed.

**Quality by Design**

Conventional approaches in the research of delivery system involving nano career, are very complex, costly and time consuming. Quality is all the time been a major apprehension for entire the pharmaceutical industries and for cultivating the quality it is desirable to be assembled up in the product. Therefore, numerous components have to be learned and assessed similar to the quality target product profile, systematic knowledge, design space and design of experiments. According to the enactment of QbD, risk assessment has the precedence over DoE [9]. Amongst the tools, Ishikawa fishbone diagram and FMEA are extensively used methodologies for risk assessment, either separately or in combination [10]. Taking the preparation of lipoplex as a two-step process, the Ishikawa diagram is shown in Figure 1 in a step-wise manner. The fishbone diagram as shown in Figure 2 classified risk factors into wide classifications, while the FMEA could recognise the failure modes that have the utmost chance of instigating product failure, which means maximum of the aspects in the Ishikawa fishbone diagrams will be categorized later in the FMEA analysis.

**Identification of quality target product profile and critical quality attributes**

For development of a robust product the Quality Target Product Profile (QTPP)
forms the basis of design [11]. In order to outline the QTPP for a product, the concerns to be involved are the dosage, route of administration, stability, release characteristics and various physicochemical properties affecting the quality of product. To establish the expected quality of the product, the critical quality attributes are desirable to be accredited such that the characteristics of the final product are within
the distinct suitable choice. Well ahead critical process parameters and material characteristics heart-rending the Critical Quality Attributes (CQAs) are recognised and arranged by collaborating process QRM and were estimated to what magnitude their deviation had an influence on the superiority of the product.

**Risk assessment by FMEA**

FMEA is a tactic to categorise the failures, effects and hazards in a process or product and then eradicate or diminish them. FMEA is a qualitative and methodical tool, typically generated within a database, to support specialist’s expectations what might be mistaken with a product or process. In addition to recognising how a product or process might be unsuccessful and the effects of that failure, FMEA also aids in finding the potential sources of failures and the possibility of failures being spotted before incidence. A complete risk evaluation is portrayed in the Ishikawa diagrams that comprehend all the process parameters that can impact or yield a failure regarding the quality of the final product [12]. FMEA method sanctions to empathise and prioritising the failure modes that are utmost likely to cause product failure. The prioritization trails following measures: Regularity of occurrence, severity of effects, and trouble of detection. Evaluation of respected elements was accomplished on an extent from 1 to 5, as follows: The occurrence (O) was categorised as 5 for frequent, 4 for probable, 3 for occasional, 2 for remote, and 1 for improbable; the severity (S), implicate the concerns of the failure mode, was categorised as 5 for catastrophic, 4 for critical, 3 for serious, 2 for minor, and 1 for negligible; to conclude, the third criterion, the detectability (D), denotation the trouble to recognise the failure mode, was ordered as 5 for hard to detect, 4 for low chance to be detected, 3 for moderately detectable, 2 for highly detectable, and 1 for easily detectable. The three aspects were evaluated for each of the deliberated CPPs. The failure risk was considered as Risk Priority Number (RPN) = O × S × D [13].

Specifically, three major Quality attributes, particle size, particle size distribution/Particle Size Index (PDI) and complexation efficiency, were defined and further delineated to identify potential risks. After the analysis, eight key variables were identified for screening in subsequent studies to identify all potential risks. After the analysis, eight key variables were identified for screening in subsequent studies.

The relative risk that each drug substance attributes presents was ranked according to Risk Priority Number (RPN). The highest ranked CPPs, explored in the preliminary experiments were further optimised using experimental design. Whereas, CPPs following the highest ranked, were optimised using OVAT principle.

The classical optimization technique which includes altering One Variable at A Time (OVAT) while keeping all the other variables constant level is used for the optimization of low to medium risk priority factors [14]. In another word, process parameters are the parameters of low to medium risk priority that is optimised by OVAT optimization technique while DOE tool was used for the optimization of formulation parameters (lipid components and their concentration) that are classified as high risk priority factors. The DOE tool plays an important role in studying the complexity of the pharmaceutical formulations by making the use of established statistical tools such as factorial designs [15].

**Optimization using DOE**

In general, DoE is a concept that uses a desired set of experiments to optimize or investigate a studied object. Liposomal formulation was optimized for size and PDI using D-optimal design with total 19 runs among which 6 model points were for pre-selected quadratic model, 5 points to estimate the lack of fit, 5 replicate points and additional 3 center points to evaluate for curvature and to estimate the pure error. From the results of optimum batch observed in preliminary screening, molar ratio of DOTAP (positively charged lipid) was kept constant i.e. 20 mole % and mPEG 2000-DSPE level of 1 mole % in experimental design for all batches while varying the mole % of other lipids i.e. HSPC, DPPE and Cholesterol. Chosen variables for optimization; coded and actual values for optimization are tabulated here in Table 1. Furthermore,
the design was also constrained so as to retain total molar concentration of three chosen lipids to be 80 mole % in each combinations. Additionally, process related parameters were also kept constant throughout the optimization process.

19 batches of liposomes were prepared using composition depicted in the design matrix in Table 2. All formulations were evaluated for particle size and Polydispersity Index (PDI) and the results obtained are shown in the same table. All experiments were replicated three times and mean values of experiments were fed to the design matrix for statistical evaluation.

**Table 1:** Constrains and coded values for design components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Name</th>
<th>Units</th>
<th>Type</th>
<th>Low Actual</th>
<th>High Actual</th>
<th>Low coded</th>
<th>High coded</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>HSPC</td>
<td>mole %</td>
<td>Mixture</td>
<td>50</td>
<td>65</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>V2</td>
<td>Cholesterol</td>
<td>mole %</td>
<td>Mixture</td>
<td>5</td>
<td>17.5</td>
<td>0</td>
<td>0.833333</td>
</tr>
<tr>
<td>V3</td>
<td>DPPE</td>
<td>mole %</td>
<td>Mixture</td>
<td>10</td>
<td>20</td>
<td>0</td>
<td>0.666667</td>
</tr>
</tbody>
</table>

Design constrains (V1+V2+V3) = 80 mole %

**Table 2:** Design matrix for cationic liposome optimization.

<table>
<thead>
<tr>
<th>Std</th>
<th>Run</th>
<th>V1: HSPC Mole %</th>
<th>V2: cholesterol mole %</th>
<th>V3: DPPE mole %</th>
<th>Size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>1</td>
<td>54.419</td>
<td>5.581</td>
<td>20.000</td>
<td>225</td>
<td>0.756</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>57.623</td>
<td>12.377</td>
<td>10.000</td>
<td>203</td>
<td>0.367</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>50.662</td>
<td>9.337</td>
<td>20.000</td>
<td>190</td>
<td>0.245</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>54.419</td>
<td>5.580</td>
<td>20.000</td>
<td>231</td>
<td>0.749</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>53.764</td>
<td>13.971</td>
<td>12.264</td>
<td>176</td>
<td>0.174</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>50.662</td>
<td>9.337</td>
<td>20.000</td>
<td>194</td>
<td>0.546</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>60.350</td>
<td>5.000</td>
<td>14.649</td>
<td>211</td>
<td>0.961</td>
</tr>
<tr>
<td>13</td>
<td>8</td>
<td>55.564</td>
<td>10.161</td>
<td>14.274</td>
<td>138</td>
<td>0.191</td>
</tr>
<tr>
<td>17</td>
<td>9</td>
<td>50.689</td>
<td>17.500</td>
<td>11.810</td>
<td>241</td>
<td>0.387</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>55.033</td>
<td>9.724</td>
<td>15.242</td>
<td>145</td>
<td>0.392</td>
</tr>
<tr>
<td>14</td>
<td>11</td>
<td>55.564</td>
<td>10.161</td>
<td>14.274</td>
<td>132</td>
<td>0.199</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>50.689</td>
<td>17.500</td>
<td>11.810</td>
<td>225</td>
<td>0.175</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
<td>55.564</td>
<td>10.161</td>
<td>14.274</td>
<td>135</td>
<td>0.214</td>
</tr>
<tr>
<td>11</td>
<td>14</td>
<td>58.212</td>
<td>8.921</td>
<td>12.865</td>
<td>152</td>
<td>0.531</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>57.623</td>
<td>12.377</td>
<td>10.000</td>
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<td>0.472</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>50.000</td>
<td>13.377</td>
<td>16.629</td>
<td>199</td>
<td>0.214</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>61.180</td>
<td>8.589</td>
<td>10.230</td>
<td>151</td>
<td>0.364</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>64.999</td>
<td>5.001</td>
<td>10.000</td>
<td>203</td>
<td>0.871</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>64.999</td>
<td>5.001</td>
<td>10.000</td>
<td>205</td>
<td>0.756</td>
</tr>
</tbody>
</table>

**Table 3: Quality Target Product Profile (QTPP) of lipoplex.**

<table>
<thead>
<tr>
<th>Quality Target Product Profile (QTPP)</th>
<th>Quality target product profile</th>
<th>Inference</th>
<th>Justification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dosage form</td>
<td>Liquid dispersion</td>
<td></td>
<td>To ensure patient compliance and better efficacy</td>
</tr>
<tr>
<td>Route of administration</td>
<td>IV injection/intranasal</td>
<td></td>
<td>To improve efficacy of formulation</td>
</tr>
<tr>
<td>Particle size</td>
<td>&lt; 200 nm</td>
<td></td>
<td>To avoid necrosis/irritation at the site of injection or to improve absorption in nasal route</td>
</tr>
<tr>
<td>PDI</td>
<td>&lt; 0.300</td>
<td></td>
<td>To ensure uniform size distribution</td>
</tr>
<tr>
<td>Phospholipid assay</td>
<td>&gt; 80%</td>
<td></td>
<td>To ensure consistency in production</td>
</tr>
<tr>
<td>Complexation efficiency</td>
<td>&gt; 95%</td>
<td></td>
<td>To ensure consistency in production</td>
</tr>
<tr>
<td>Stability</td>
<td>Short term stability of 3 months on accelerated condition 40 °C/75% RH and 3 months long term conditions 25 °C/60% RH</td>
<td>Minimum time period (at least 3 months initially) decided to study stability of final formulation</td>
<td></td>
</tr>
</tbody>
</table>

the design was also constrained so as to retain total molar concentration of three chosen lipids to be 80 mole % in each combinations. Additionally, process related parameters were also kept constant throughout the optimization process.

19 batches of liposomes were prepared using composition depicted in the design matrix in Table 2. All formulations were evaluated for particle size and Polydispersity Index (PDI) and the results obtained are shown in the same table. All experiments were replicated three times and mean values of experiments were fed to the design matrix for statistical evaluation.

**Result and Discussion**

The first (and the most significant) component in using the QbD idea to support formulation and process design is to predefine the anticipated ultimate product quality profiles. This study dedicated on critical formulation qualities, namely particle size, PDI and complexation efficiency.

**QTPP of lipoplex**

Laying down QTPP depends upon formulation type and process chosen. Built
on initial trials commenced, the parameters that will be attentive in our work were designated and recruited as QTPP lipoplex (Table 3). Accordingly, excluding performance of our QTPP, the additional steps to designate the QTPP are not conferred. The said QTPP will lay down the basis for decisive CQA.

Risk assessment by FMEA

The factors that were embarked and assessed by FMEA in development of lipoplex formulation are highlighted in Table 4. In the present method for development, the factors that exhibited RPN count above 50 was considered as high risk, 20 to 40 was considered as medium risk and 20 was considered as low risk. From Table 4 and Table 6, it is clearly stipulated that selected lipids and their mole % had RPN over 50 and require detailed investigation and optimization. Thus, the optimization of main factors that affect liposome i.e. the used lipids and their amount was done statistically using D-Optimal design for establishing design space. RPN for remaining factors fall under moderate (below 50) to low (below 20) risk category.

Contrasting to typically established designs such as factorials and fractional factorials, D-optimal design matrices are regularly not orthogonal and effect assessments are related [16]. The reasons for using D-optimal designs instead of standard classical designs generally fall into two categories:

1. Standard factorial or fractional factorial designs require too many runs for the amount of resources or time allowed for the experiment.
2. The design space is constrained (the process space contains factor settings that are not feasible or are impossible to run).
DOE for liposome

Response surface modelling was applied using Design Expert 9.0.1 (Stat-Ease Inc., MN). Using Multiple Linear Regression Analysis (MLRA), different polynomial equations were evaluated for best fitting to the experimental data by determining the values of coefficients in the polynomial equations and a full and reduced model was established. Statistical soundness of the established model was checked by ANOVA statistics.

Based on the established model three dimensional response surface plots were constructed by Design Expert Software. The 3D surface plots were useful in establishing the main effects (effect of individual variables) on the response parameter and also to have an insight to the combined effects of two variables [17] enlisted in Table 1 design constrains.

Validation of the employed experimental design and chosen model for its prediction capability for the optimization of the variables was done by performing checkpoint analysis [18]. Eight optimum checkpoints were selected, prepared and evaluated for response parameters. Statistical comparison between the predicted values and average of three experimental values of the response parameters was performed to derive percentage error and to evaluate significant difference between these values. Optimum formulation parameters were selected based on the specified goal i.e. particle size and particle size distribution. Optimized formulation was derived by specifying goal and importance to the formulation variables and response parameters. Results obtained from the software are shown in Table 2 and further verified by actual preparation of the batches and comparing the predicted and actual results.

### Statistical Analysis Response

#### Selection of the predicted model

Summary of the ANOVA results for different models portrays sequential p-values and Lack of Fit p-values along with Adjusted and Predicted R-squared values for different models for both size and PDI shown in Table 7.
Highest polynomial displaying highest Lack of Fit p-value for size and PDI were 0.5965 and 0.3350 respectively, i.e. > 0.1, was considered for model selection which is shown in Table 8. Based on the criteria, for size, special cubic model
and for PDI quadratic model was found to be best fitted to the observed responses. Other models were not suitable for prediction either due to low R-squared values and/or due to higher p value as contrasted to other models. As shown in Table 9 for the analysis of variance, the model F-values of 24.41 and 12.30 values respectively for size and PDI, imply that the model is significant. There is only a 0.01% chance that a “Model F-Value” this large could occur due to noise. Values of “Prob > F” less than 0.0500 indicate model terms are significant. For size, AB, BC, ABC and for PDI Linear Mixture Components, AB, BC are significant model terms. If there are many irrelevant model terms (not including those mandatory to support hierarchy), model reduction may advance model. The “Lack of Fit F-value” of 0.78 and 1.38 separately for size and PDI, implicit the Lack of Fit is not significant comparative to the pure error. There were 59.65% and 33.64% respective chances for size and PDI that a “Lack of Fit F-value” this big could take place due to noise.

Final equation in terms of actual components for size is

\[
\text{Size} = - (1.94453 \times \text{HSPC}) - (59.57715 \times \text{Cholesterol}) - (43.45504 \times \text{DPPE}) + (1.7559 \times \text{HSPC} \times \text{Cholesterol}) + (1.23787 \times \text{HSPC} \times \text{DPPE}) + (13.42311 \times \text{Cholesterol} \times \text{DPPE}) - (0.30373 \times \text{HSPC} \times \text{Cholesterol} \times \text{DPPE})
\]

In a same way, final equation in terms of actual components for PDI is

\[
\text{PDI} = (1.80155 \times \text{HSPC}) + (31.42788 \times \text{Cholesterol}) + (11.19406 \times \text{DPPE}) - (42.09090 \times \text{HSPC} \times \text{Cholesterol}) - (12.67092 \times \text{HSPC} \times \text{DPPE}) - (72.72618 \times \text{Cholesterol} \times \text{DPPE})
\]

ANOVA results for special cubic and quadratic models from Table 10 suggested the “Pred R-Squared” of 0.8029 and 0.5957 for size and PDI were in moderately harmony with the “Adj R Squared” of 0.8864 and 0.7584 i.e. < 0.2. “Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. Consequently, ratios of 13.529 and 9.844 indicated an adequate signal. This model can be used to navigate the design space for size and PDI. When accompanying any statistical analysis it is imperative to assess how well the model fits the statistics and that the statistics meet the expectations of the model. There are frequent ways to do this and a multiplicity of statistical tests to assess deviations from model expectations.

Various diagnostic plots for evaluation of the particle size model are shown in Figure 1 and same for the PDI, are shown in Figure 3. The normal probability plot (normal plot of residuals) which shows whether the residuals follows a normal distribution or not and helps identify any specific patterns in the residuals indicative of requirement of transformations i.e. “S-shaped” curve, etc. normal plot for the current data show that data follows a straight line inferring the normal distribution of the residuals. It is a scatter plot composed by residuals on the y axis and x axis limited to the fitted values (estimated responses). It is used to identify non-linearity, unequal error variances, and outliers [19]. Residuals vs. predicted response (ascending values) plot tests if variance is distributed over the design constantly i.e. variance is not associated with the factor values as megaphone patterns (pattern like sign > or <) which indicate either decreasing or increasing residuals with increasing the factor values. This is a plot of the residuals versus the ascending predicted response values.

Residual vs. run order plots the residuals against the experimental run order i.e.
order in which the experiments have been carried out [20]. The plot with a random scatter of residuals indicate there is no time dependent changes in the residuals, i.e. there is absence of any time dependent variable among the variables selected or among the variables which has been not included in the DoE. If there is such Random scattered plot of residuals in the current analysis indicate no association of residuals with time and absence of any time dependent variable. Predicted vs. actual plot shows correlation between the observed response values and actual response values. A good correlation between the predicted and actual values (data following a straight 45° line) shows that the model chosen for analyses of data is appropriate in predicting the responses all over the design matrix [21]. Also, this plot helps to detect a value/values that are not easily predicted by the model. As it can be seen from the Figure 1 and Figure 3, plot follows a 45° straight line indicating a close estimate of predicted values with actual values. A Box Cox transformation is a way to transform non-normal dependent variables into a normal shape [22]. Many statistical tests and intervals are based on the assumption of normality. The hypothesis of normality frequently primes to tests that are simple, statistically controllable, and influential compared to tests that do not make the normality supposition. Unfortunately, many actual data sets are in fact not nearly normal. Nevertheless, a suitable alteration of a data set can often yield a data set that does trail around a normal distribution. This upsurges the applicability and practicality of statistical techniques based on the normality supposition. Normality is an important assumption for many statistical techniques; if data isn’t normal, applying a Box-Cox means a broader number of test can be applied. Box-Cox plot of Ln (residuals sum of squares) vs. λ for power transformation helps select any power transformation of observed response values required for fitting the model based on the best λ value and 95% confidence interval around it. Plot in Figure 4A shows the λ value of 1, which lies near the best

Figure 3: Model diagnostic plots for PDI.
λ value and within 95% confidence interval of it, indicating no requirement for any power transformation.

Piepel’s plot is a trace plot showing the effect of individual factors plotting the pseudo limits of one component keeping the ratio of the changeable amounts of each component kept constant against the response [23]. The responses are plotted as deviations from the reference blend i.e. centroid (pseudo center point of the constrained design). As it is depicted in plot from Figure 4B, HSPC, cholesterol and DPPE, all of them have negative effects on particle size up to a certain point, indicated by decrease in particle size, afterwards, they have positive effect on the particle size indicated by increase in particle size by increasing mole %. For PDI, HSPC and cholesterol, have the more impact on that of DPPE; give wide range of PDI with change in mole % of HSPC or cholesterol.

Two component mix effects

In the case of size, two component mix plots in Figure 5 show the combined effects of two components on the response keeping the value of another component kept constant at its centroid value. As the ratio of HSPC: DPPE increases up to a point, with invariable mole % of cholesterol, particle size diminishes; after that point increase in ratio of HSPC: DPPE increases, particle size also increased. The size deviations, in turn, is explained by the relative small area per lipid adopted by PE lipids versus the PC lipids. Additionally, the inner monolayer favours inverted-cone shaped lipids, the fraction of DPPE is enhanced in the inner monolayer as DPPE possesses inverted-cone shape, leading to a more symmetric overall lipid distribution at lower HSPC: DPPE ratio, overcompensating for the size effect of HSPC [24]. PC lipids tend to arrange themselves in very tight packing arrangement, so increase in mole % of HSPC helps in reducing size of vesicles. But after a certain point, it also appears that the PC lipids tend to shift a little more toward the bilayer interfaces, whereas the PE lipids found more of the interior space. This effect arising from the larger tendency of PC lipids to be hydrated in comparison to the PE lipids which form intra-lipid hydrogen bonds more easily thus at

![Box-Cox and Trace plots for A) Particle size; B) PDI.](image-url)
higher HSPC level, more lipid tends to be at outer interface leading to size increment with increase in HSPC level [25]. At higher HSPC: DPPE ratio, these phenomena shows prominent effect on size leading to increase in liposomal size.

Cholesterol doesn’t form a bilayer itself, but it gets dissolved in the phospholipid bilayer. After a point, at higher concentration of cholesterol, irrespective of mole % of another component, additional cholesterol molecules will be dispersed in the phospholipid bilayer, producing a rise in the liposome mean diameter. At higher mole % of cholesterol integration into phospholipid bilayers, the small hydrophilic 3 β-hydroxyl head group of cholesterol is found in the locality of the lipid ester carbon-yl groups, and the hydrophobic steroid ring positions itself parallel to the acyl chains of the lipid. Thus, the movement of the acyl chains of the phospholipid bilayer has been restricted fetching the motive for let-down of constricted packing arrangement of other lipid components. It has been reported that higher cholesterol concentrations interfere with the close packing of the phospholipid bilayer by contributing to an increase in membrane fluidity which results in an increased distribution of aqueous phase within the liposomal vesicles [26]. This explains the direct increase

Figure 5: Two component mixture plots for A) Particle size; B) PDI.
of liposomes mean diameter observed with higher cholesterol: HSPC ratio. Then as cholesterol: HSPC ratio decreases, size of the vesicles also decreases because of increasing mole % of HSPC resulting in tight packing of vesicles, up to a certain point. After that point, as mole % of HSPC increases, irrespective of mole % of cholesterol, HSPC tend to aligned themselves at outer surface of vesicles that ultimately, increases the size of the liposomal vesicles. The same reasons are responsible for the size effects of DPPE: Cholesterol mole ratio on liposomes at invariable mole % of HSPC.

For PDI, as the ratio of Cholesterol: HSPC and Cholesterol: DPPE increases i.e. by increasing the mole % of cholesterol leads to lower PDI. The obtained results could also be explained based on the membrane rigidity resulted from CHO inclusion. It is well recognised that integration of CHO conveys firmness to the bilayer membrane, thus progress the physical stability for liposomal system. Additionally, CHO can stabilize the bilayer structure by eliminating the phase transition temperature peak of the vesicles, thereby strengthening the bilayer structures and diminishes bilayer micro fluidity. At low concentration of CHO, the vesicular membranes are more flexible and more liable to the effect of distortion. With increasing CHO concentration, the rigidity of the membranes increased with increased resistance to change in size, thus constructing vesicles with uniform size distribution.

Figure 6: Contour and response surface plot for A) Particle size; B) PDI.
Contour plot and response surface plot show effects of all three components on the particle size in Figure 6A and on PDI in Figure 6B. To summarize, with increasing mole % of individual component brings about increment in size of liposomes up to a point after which increasing mole %, shows diminished liposome size. While in case of particle size distribution, the effects can be explained with similar justifications mentioned under the two component mix plots also.

**Selection of formulation parameters**

Constraints applied to select the best formulation parameters based on the desired particle size and polydispersity index.

All the affecting factors were to be optimized within the range chosen for design matrix. Particle size was to be optimized at the minimum value possible in the range observed experimentally (130-150 nm) and PDI was to be optimized at the minimum value possible in the experimentally observed range of 0.174-0.350. The optimization was grounded on the desirability criteria which makes the superlative trade off amid the constrains and choosing a group which gratifies the criteria, the best for optimization and weighs the prediction based on a desirability index which ranges from 0 (for the least suited combination) to 1 (the best suited combination). The desirability plot which represents the desirability index over the design is shown in Figure 7 showing a flag where the optimized batch lies.

Based on the maximum desirability, one formulation (desirability 0.546) was found to best fit the selection constraints. Predicted responses of this batch were 140.73 nm particle size and 0.250 PDI.

Additionally, selection criterial was also applied in order to select the design space within the design matrix where desired formulation responses can be observed. The
selection criteria were particle size range of 100-125 nm and PDI of 0.150-0.200 and based on these criteria, following design space was found.

**Point prediction and confirmation**

Table 11 shows predicted response for the solution selected above along with the Standard deviation and 95% confidence interval of the response. Confirmation of the response was done by carrying out the experiment using the selected factor values in triplicate shown in Figure 8.

Table 12 shows and confirms that experimental and predicted values are in good agreement confirmed by calculating p values for both size and PDI. Results showed values of p < 0.05, indicated no significant difference existed between practical value and standard value, concluding the suitability of the selected model for optimization.

**Optimization of lipoplex**

**Incubation time:** It is well recognised that DNA-liposome complex aggregates and increases in particle size upon storage, indicating structure changes in the complex with time [27]. We thus examined if the serum sensitivity of the complex would also change with time of incubation in period of 10 to 50 minutes. DNA-liposome complex was incubated for different lengths of time at room temperature after mixing liposomes with DNA at a theoretical charge (+/−) ratio of 2.0 at which maximum complexation took place. At the end of incubation, fetal bovine serum (final concentration 20%) was added to the complex. As shown in Figure 9, as the incubation time increased, the sensitivity to serum gradually diminished. The complex had become essentially serum resistant after 40-50 min of incubation. These results indicate that the DNA-liposome complex had undergone a maturation process with time which leads to its total resistance to serum.

**Incubation temperature:** In order to investigate the effect of temperature on complexation efficiency of lipoplexes, incubated with cDNA at different temperatures i.e. at 20 °C ± 2 °C, room temperature 37 °C ± 2 °C temperature (RT) and 55 °C ± 2 °C temperature for 45 min of incubation period. The Tg (glass transition temperature) of lipids used in the formation of lipoplex as well as varied mobility of cationic liposomes and cDNA at different temperature play a crucial part in the complexation efficiency. It was observed that for a constant incubation period, temperature affected the complexation efficiency of liposome as shown in Figure 9. For 45 minutes incubation, the complexation efficiency at L/P ratio which gave maximum complexation, there was no change found at 37 °C and 55 °C for all the formulations but drastic change was found in complexation efficiency at 20 °C temperature. This is most likely attributed to change into more disordered liquid state from the tightly packed gel phase of lipid at temperature higher than Tg of lipids used in the formulation. HSPC and DPPE are the saturated lipids, with higher Tg of around 55 °C and 41 °C respectively, used in this formulation in quantities more than 70 mole % of total lipid content. Therefore, the liposomes formed from these lipids require higher temperature for the gel to liquid transition for more efficient permeation of cDNA molecules into liposomal vesicle which ultimately resulting in more complexation of cDNA to liposomes. Moreover, the decreased mobility of cDNA molecules rendering the flexibility in the cDNA structure to approach the cationic surface [28]. Also, decreasing temperature also decrease the movement of PEG chains extending on the surface of liposomes which allows closer approach of the cDNA nearer to the cationic charge bypassing the PEG chains.

**pH:** PEs (Phosphatidylethanolamines) is not completely zwitterionic in nature at all pH. Phosphatidylethanolamine carry two charges which in a mixed phospholipid bilayer, strong intermolecular hydrogen bonding to an immediate phospholipid. This forms an ion pair between the positively charged amine of one phospholipid and the negatively charged phosphate of a neighbouring phospholipid. This close favourable interaction greatly diminishes the polar character of the headgroup at neutral pH. Because the pKₐ of the PE amine is about 8.5, at alkaline pH the amine present in PEs is only partially protonated. With the fully dissociated phosphate ions, PEs have
a slightly negative net charge at alkaline pH. Herein, the optimised formulation comprised of two PEs, DOPE and DPPE, consequently acquire negative charge at alkaline pH, and in due course affect the overall positive charge possessed by liposomal vesicle. This ultimately results in diminished ability of negatively charged DNA molecules towards the liposome and showed difference in complexation efficiency at alkaline pH than that of acidic and neutral pH as shown in Figure 9.
Conclusion

High manufacturing inconsistency as a consequence of lack of indulgent in the preparation process means a much more rigorous evaluation process essential for the safety concern. As a result, it is the unbiased existing study to exploit the QbD principles to support formulation process design, to aid in understanding the foundations of the variability to mend the product quality. To achieve this, p11 cDNA was used as a model compound. Preferred profiles for key product qualities, namely particle size, PDI and complexation efficiencies, were defined and assessed. The goals were to attain as high as possible the complexation, while upholding the size and size distribution as narrow as possible. It was perceived that the liposome preparation process parameters and formulation parameters, has massive impact on aforementioned quality attributes. However, through applicable process design, very reliable particle size, its distribution and complexation efficiency can be accomplished. It is revealed that lipid selection and its ratio has the leading effects of liposome size and PDI which was optimised using D-optimal design. After formation of cationic liposome, it was complexed with negatively charged cDNA which was affected by pH, incubation time and incubation temperature. Finally, the use of risk assessment backed the identification of high risk factors that have influences on quality attributes. This information will be beneficial for a more ample experimental design to better recognise the connections among the variables and to acquire the appropriate design space.

Conflict of Interest

Authors report no conflict of interests and are accountable for the content of the research paper.

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PE is a primary amine-containing phospholipid and therefore has a highly reactive chemical handle that can be easily derivatized. This property will be exploited in Chapter 9 for various membrane biochemical studies. Because the pKa of the PE amine is about 8.5, at physiological pH the amine is mostly, but not completely, protonated. With the fully dissociated phosphate, PEs have a slightly negative net charge. PEs are therefore not quite a full zwitterion. PE’s primary role in membranes is as a major structural lipid. PE’s head group is not only chemically reactive but also small and poorly hydrated.

References


