Formulation and evaluation of liposomes containing antitubercular drugs by Taguchi's orthogonal array design

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Abstract

Isoniazid (INH) and rifampicin (RIF) are effective drugs for treatment of tuberculosis (TB). Considering effectiveness of combine drug therapy of INH and RIF, the study focused on formulation of liposomes. The objective of this study was to achieve desired entrapment of antitubercular drugs (rifampicin and isoniazid) in liposome. The liposomes were produced by thin film evaporation technique using soya phosphatidylcholine. The evaluation studies of different processing variables like drug to lipid ratio, phosphatidyl choline to cholesterol ratio, temperature and hydration time were evaluated by Taguchi design on percentage drug entrapment (PDE), size distribution, release rate, particle size. Liposomes showed encapsulation efficiency of 79.69%± 2.260 for RIF and 47.54% ±1.19 for Isoniazid. Liposomes containing both Rifampicin and Isoniazid were optimized based on the individual optimized formulation. Liposomes obtained have average vesicle size of 14.66 μm in case of single drug liposome and 18.19 μm in case of liposomes containing both drugs (INH AND RIF). Thus it was possible with Taguchi OA design of optimization to prepare liposome containing hydrophilic and hydrophobic.

Key words: liposomes, antitubercular drugs, multilamellar vesicles, lyophilization, drug release.

Introduction

Isoniazid (INH) and rifampicin (RIF) are effective drugs for the treatment of tuberculosis. Although the mechanism of action of INH is not clearly known there is evidence that it inhibits the synthesis of mycolic acid, an essential component of the bacterial cell wall, and also combines with an enzyme that is uniquely found in INH-sensitive strains of mycobacteria. Resistance to INH can occur due to reduced intracellular penetration of the drug. On the other hand, RIF acts by binding to, and inhibiting, DNA dependent RNA polymerase (Rang et al. 1999). Rifampicin is the first choice drug in the treatment of tuberculosis but requires a high-dose drug treatment over a period of 4–6 months. The causative organism is known to develop resistance with conventional delivery systems as serum levels were found to fluctuate below minimum inhibitory concentrations in clinical investigations (Mandell and Sande 1985). Since resistance occurs in one drug therapy, treatment with combination of these drugs is recommended. Between the various types of drug delivery systems proposed, liposomes have many advantages, as their suitability for lipophilic drugs and the fact that they have potential for prevention of local irritation, increased potency and reduced toxicity (Gilbert et al. 1991).

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Liposomal aerosols in pulmonary therapy offer the additional advantage of uniform deposition of locally active drugs (Gilbert et al. 1991, Parthasarathy et al. 1999). RIF was selected also because it is a first choice drug for tuberculosis treatment (Mandell and Sande 1985, Rang et al. 1999) and resistance to RIF can develop rapidly (Rang et al. 1999). Thereby, there is a therapeutic rational for delivering RIF with liposomes, in order to passively target alveolar macrophages where a large number of tubercle bacilli harbour (Bermudez 1994). Liposomes are used for effective medication by enclosing an aqueous solution with a membrane of phospholipids. Encapsulating a sufficient amount of the therapeutic agent is one of the most desirable properties for their usage (Sharma and Sharma 1997, Heurtault et al. 2003, Mainardes and Silva 2004). The liposomes encapsulate a hydrophilic drug within an aqueous component, while liposomes also entrap the lipophilic drug within the lipid bilayers (Barenholz 2003). Factors affecting the encapsulation efficiency of the drug in the liposomes are various and come from the properties of both the liposomes and encapsulated drugs. Concerning the encapsulated drugs, the encapsulation efficiency is affected by hydrophilic or lipophilic properties and tended to interact with the membrane bilayers (Kulkarni et al. 1995, Barenholz 2003). As for the liposome properties, aqueous volume, membrane rigidity, surface area (Kulkarni et al. 1995) and preparation methods (Kirby and Gregoriadis 1984) are reported to have influenced the encapsulation efficiency. Liposomes have been advocated as carrier to deliver the antibacterial to the pre-selected targets. Pressurized packed liposomes for the pulmonary targeting of drugs have been well documented (Farr et al. 1985, 1987, Vyas and Sakthivel 1994). Liposomes containing both Rifampicin and Isoniazid would offer considerable benefits by way of increased concentration of drug at site of action such as lung as well as controlled release characteristics. INH or RIF stealth liposomes with enhanced affinity towards lung tissue were prepared by modifying the surface of stealth liposomes by tagging O-stearylamlylopectin thus resulting in an increase in the affinity of these liposomes towards the lung tissue of mice (Deol and Khuller 1997). Multiple emulsions of RIF also gave a sustained release profile, and coating these emulsions with polysaccharide was found to reduce toxicity compared to the free drug (Khopade et al. 1996). Liposomes containing INH and RIF were formulated by co encapsulation method with higher entrapment efficiency for effective tuberculosis therapy (Gursoy et al. 2004). Statistically experimental design methods provide a systematic and efficient plan for experimentation to achieve certain goals so that many control factors can be simultaneously studied. The simplex method (Nakai 1984, Chen 1992), evolutionary operation (Banerji 1993, Tung 1999), response surface methodology (Houng 1989, Sreekumar 1999, Chopra 2007) and the Taguchi method (Cobb 1994, Stone 1994, Jahanshahi 2008) are frequently applied experimental design methods. The Taguchi method has the advantages of optimization of many more factors simultaneously and extraction of much quantitative information by only a few experimental trials (Bendell 1989). Optimization of the process implies the use of a designed experiment in order to: (1) identify factors affecting the procedure, (2) estimate the factor levels yielding an optimum response, and (3) decrease the process variability without controlling or eliminating causes of variation. Thus, optimizing process parameters by the Taguchi method is an attempt not only to bring the average quality near to the target value but also to simultaneously minimize the variation in quality (Houng 2003). For example, to study the effect of four variables by a full factorial design requires
conducted evaluations of 81 separate formulations however, in case of Taguchi design requires only nine. Taking into consideration the well established combined therapy of INH and RIF, this study focused on encapsulation of INH and RIF in the single liposome formulation prepared by thin film evaporation method in accordance with Taguchi OA design. INH was incorporated in the aqueous phase and RIF in the lipid layer. The variables that affect entrapment of drug were evaluated by Taguchi design.

Materials and Methods

Rifampicin and Isoniazid were generously provided as a gift sample by Strides Arcolab, Bangalore (India). Phosphatidyl choline (Soya lecithin), cholesterol were obtained from Sigma Aldrich, USA. Dialysis bag Hi-media, India. All other reagents were analytical grade and used as received.

Experimental design and analysis: Table 1 displays the four control factors selected in the optimization study. A standard orthogonal array L₉ was used to examine this four-factor system. L and subscript 9 denote the Latin square and the number of the experimental runs, respectively. The run involved the corresponding combination of levels to which the factors in the experiment was set. All studied factors had three levels and all experiments were performed in triplicate. The percentage drug entrapment of RIF and INH in liposome was considered to be the responses. The first step was to select the fact or /level combination maximize the response. The second step was to find the condition for attaining optimal desirability (Houng 2003).

Table 1. Predicted upper and lower limit of processing parameter.

<table>
<thead>
<tr>
<th>Constrains</th>
<th>Goal</th>
<th>Lower limit</th>
<th>Upper limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>D:L molar ratio</td>
<td>Is in the Range</td>
<td>1:1</td>
<td>1:10</td>
</tr>
<tr>
<td>PC: CL ratio</td>
<td>Is in the Range</td>
<td>40:60</td>
<td>60:40</td>
</tr>
<tr>
<td>Temperature</td>
<td>Is in the Range</td>
<td>37°C C</td>
<td>45°C C</td>
</tr>
<tr>
<td>Hydration time</td>
<td>Is in the Range</td>
<td>15 min</td>
<td>45 min</td>
</tr>
</tbody>
</table>

*D:L -Drug to lipid ratio, *PC: CL ratio- Phosphatidyl choline to cholesterol ratio

Preparation of drug loaded Liposomes: Multilamellar vesicles with and without the drugs were prepared by using a solvent system (CHCl3:CH3 OH [2:1], 10 mL) for a solvent stripping technique in a rotary flask evaporator. Soya lecithin and cholesterol were dissolved in chloroform: methanol (2:1) solution. Organic solvent was slowly removed under reduced pressure so that a thin film of dry lipid was deposited on the inner wall of the flask. This film was further hydrated dispersed in 10ml saline phosphate buffer pH 7.4 at 25°C for a required period of time. Rifampicin was initially mixed with lipids while isoniazid was dissolved in PBS buffer.

Lyophilization of liposomes: Liposomal dispersions were freeze dried using 1:1 ratio on weight basis of sucrose and lipid. Liposomal dispersions were prepared by hydrating a dry lipid film with aqueous phase containing sucrose. Non encapsulated drug / drugs were removed by dialysis for 30 min. The dispersion was frozen to – 40°C and freeze drying was performed in freeze drier (Lyso, Japan) till a dry powder was obtained. The whole drying process took 24 h with primary drying at – 40°C for 16 h and secondary drying at – 20°C for 8 h. Lyophilized products were further subjected to moisture content, particle size, drug content and drug entrapment studies.

Physical mixture: Physical mixtures of Rifampicin and Isoniazid individually with excipients were subjected to Infra Red (IR) spectral analysis (FTIR 8400 Spectrophotometer). The disc of KBr were prepared by pelletization method containing 1 part of drug to 100 part of KBr and subjected for interpretation.
HPLC Method: Quantitative estimation of Rifampicin and Isoniazid individually by HPLC (SPD-10 AVP) was carried out at 254 nm using Genesis C-18 column (ODS - 150 x 4.6 mm). The mobile phase was developed for estimation of Rifampicin and Isoniazid individually and in combination. Mobile phase was made up of 75% methanol and 25% buffer (0.02 M disodium hydrogen orthophosphate) adjusted to pH 4.5 by orthophosphoric acid. Isobestic point was taken at 254 nm for Rifampicin standard graph.

Optimization: In the present study, the four independent variables were evaluated which include drug to lipid ratio, phosphatidyl choline to cholesterol ratio, temperature and hydration time. The relationship between variables was elucidated by using one factor plots. A numerical optimization technique by the desirability approach was used to generate the optimum setting for the formulation using entrapment efficiency which was kept in the range.

Drug Content: For determination of drug content a 0.1% solution of surfactant, Triton X-100 in methanol to get the concentration 1mg/ml. further dilution was done with mobile phase and measured the area of injectable solution. Dialysis technique was carried out for removing unentrapped drug (RIF or INH) from drug loaded liposomal suspension to determine PDE. Dialysis membrane – 150 having a pore size of 2.4 nm was employed for dialysis.

Measurement of liposome encapsulation efficiency: The encapsulation of both the drug were measured by dialysis membrane – 150 (Hi-media, India), which having the cut off size 12,000 molecular weight and pore size 2.4 nm was selected for dialysis. Dialysis membrane was activated by soaking in Millipore water for two hours. One end of the membrane was tied with the thread and then the liposomal suspension was poured and tied with a thread. The bag is suspended in a beaker and placed on the magnetic stirrer. Samples were withdrawn at particular time intervals till the readings become constant.

\[
\text{Entrapment Efficiency} = \frac{\text{Drug present in liposomes}}{\text{Total Drug Content}} \times 100
\]

In vitro drug release study: Liposomal suspension was placed within a dialysis bag (cellulose membrane) and immerse into a vessel containing 100 ml of PBS a hot plate (Remi, India). The release studies were carried out at temperature 37 ± 1°C under mechanical stirring at 50 rpm. At fixed intervals of 30, 60 minutes and then every hr for up to 7 h samples were withdrawn from the solution and INH or RIF's content were determined by HPLC analysis.

Particle size measurement: The mean particle size and size distribution of liposomes were measured by dynamic light scattering (DLS) using Malvern Mastersizer (Hydro 2000 S units). The sample was placed in an automated dispersion unit and subjected to particle size analysis. The collimated laser beam is made incident to the suspended sample particles. The intensity signals of the different bar scattered light are processed into particle size distribution.

Characterization of Liposomes, Calorimetric studies: Differential scanning calorimetry (DSC) was used to determine the phase transition temperatures of phospholipid samples in order to characterize the physical state of drug. The transition temperatures correspond to the peaks of the endotherms during the heating scans. Samples consist of known mass of liposomal formulation were placed in aluminium pans and thermatically sealed. The heating rate was 10°C per minute using nitrogen as the purge gas. The DSC instrument was calibrated for temperature using Indium. In addition, for enthalpy calibration Indium was sealed in aluminium pans with sealed empty pan as a reference.

Morphological characterization: The morphology of the particles was examined using Joel 840 A, Japan, scanning electron microscope. The lyophilized liposomes were scattered individually onto a thin film of epoxy resin and coated with a platinum layer. SEM was carried out for optimized lyophilized liposomes of Rifampicin, Isoniazid and combination of both drugs.
Result and Discussion

Targeting liposomes bearing Rifampicin and Isoniazid to the lungs and specifically, to the pulmonary alveolar macrophages by aerosolization, is a promising and feasible strategy for treating mycobacterial infections. Here the formulations of liposome were carried out under taguchi design plot. This design effectively reduces the number of experiments required to carry out based formation orthogonal array set up of four different variables. Liposomes were prepared in 9 batches for each drug (INH/RIF) by thin layer evaporation technique according to Taguchi design plot taking four independent variables in account. In the present study, the four independent variables which were evaluated for liposome containing both the drugs (RIF an INH) are as follow: Factor A: Drug to lipid ratio (X₁), Factor B: Phosphatidyl Choline to Cholesterol ratio (X₂), Factor C: Temperature (X₃), Factor D: Hydration time (X₄) on percentage drug entrapment in liposomal formulation. The upper and lower limits of prediction for formulation design are shown in Table 1. The ratio of drug to lipid was varied for both the drugs (RIF and INH). In case of Rifampicin, the ratios taken were 1:1, 1:5, 1:10 while incase of Isoniazid it was 1:1, 1:2.5, 1:5. The ratio of phosphatidyl choline to cholesterol was also varied; 40:60, 50:50 and 60:40 were studied for both the drugs. The solvent evaporation was carried out at different temperature 37, 40 and 45°C. The percentage drug entrapment (PDE) was calculated at 15, 30 and 45 min of hydration time. The relationship between the variables was elucidated by using one factor plots (Taguchi OA design). According to the Taguchi OA for optimization, the design summary and response data for Rifampicin and Isoniazid is shown in Table 2.

Table 2. Taguchi design for screening of process parameters for the preparation of Liposomes containing RIF- INH.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Factors</th>
<th>RESPONSE PDE (%)</th>
<th>% Drug content for RIF</th>
<th>% Drug content for INH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X₁</td>
<td>X₂</td>
<td>X₃</td>
<td>X₄</td>
</tr>
<tr>
<td></td>
<td>RIF</td>
<td>INH</td>
<td>RIF</td>
<td>INH</td>
</tr>
<tr>
<td>1</td>
<td>1:1</td>
<td>1:1</td>
<td>40:60</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>1:1</td>
<td>1:1</td>
<td>50:50</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>1:1</td>
<td>1:1</td>
<td>60:40</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>1:5</td>
<td>1:25</td>
<td>40:60</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>1:5</td>
<td>1:25</td>
<td>50:50</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>1:5</td>
<td>1:25</td>
<td>60:40</td>
<td>45</td>
</tr>
<tr>
<td>7</td>
<td>1:10</td>
<td>1:5</td>
<td>40:60</td>
<td>37</td>
</tr>
<tr>
<td>8</td>
<td>1:10</td>
<td>1:5</td>
<td>50:50</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>1:10</td>
<td>1:5</td>
<td>60:40</td>
<td>45</td>
</tr>
</tbody>
</table>

X₁: Drug-to-lipid ratio, X₂: Phosphatidyl Choline to cholesterol, X₃: Temperature: 37°C, 40°C, 45°C, X₄: Hydration time: 15 min, 30 min, 45 min.

All batches were evaluated for PDE and mean particle diameter. The ANOVA results for entrapment efficiency are shown in Table 3, a significant (p < 0.05) variation in PDE was observed in liposomes containing rifampicin and Isoniazid. With optimized parameter liposome were again prepared and lyophilized. In second set the liposomes containing both the drugs were prepared and lyophilized. All the formulations were subjected for particle
characterization, percentage drug entrapment and drug content. The PDE was found to vary from a minimum of 43.65 % to a maximum of 84.02 % for Rifampicin and 9.10 % to 59.0% for INH. The average PDE of optimized liposomal formulation containing RIF and INH was found to be 79.69 ± 2.260 and 47.54 % ± 1.19 respectively. The drug content and percentage drug entrapment for both the drugs are shown in Table 2.

Table 3. Optimized liposomal formulation for RIF and INH with PDE achieved.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Drug</th>
<th>D:L ratio</th>
<th>PC: CHOL ratio</th>
<th>Temp °C</th>
<th>Hydration time (min)</th>
<th>Desirability</th>
<th>Response PDE</th>
<th>Predicted PDE</th>
<th>PDE for lyophilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RIF</td>
<td>1:5</td>
<td>40:60</td>
<td>40</td>
<td>15</td>
<td>1</td>
<td>81.58</td>
<td>79.69 ± 2.26</td>
<td>81.71</td>
</tr>
<tr>
<td>2</td>
<td>RIF</td>
<td>1:5</td>
<td>50:50</td>
<td>40</td>
<td>15</td>
<td>1</td>
<td>77.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>RIF</td>
<td>1:5</td>
<td>60:40</td>
<td>40</td>
<td>15</td>
<td>1</td>
<td>80.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>INH</td>
<td>1:5</td>
<td>60:40</td>
<td>37</td>
<td>45</td>
<td>0.88</td>
<td>47.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>INH</td>
<td>1:5</td>
<td>60:40</td>
<td>40</td>
<td>45</td>
<td>0.88</td>
<td>46.69</td>
<td></td>
<td>51.02</td>
</tr>
<tr>
<td>6</td>
<td>INH</td>
<td>1:5</td>
<td>60:40</td>
<td>45</td>
<td>45</td>
<td>0.88</td>
<td>48.91</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*D:L = drug to lipid ratio, PC: Chol = phosphatidylcholine to cholesterol ratio, PDE = percentage drug entrapment. † this variable does not have significant effect on overall liposomal preparation.

The vesicle size distribution of liposome gives mean diameters ranging between 3.36 and 10.90 μm. The mean vesicle size of empty and drug loaded liposomal dispersion was 5.89 μm and 7.29 μm, respectively. Additionally, empty PC/Chol liposomes prepared under identical conditions (with the corresponding RIF-entraping vesicles) were significantly smaller compared to the vesicles that incorporate the drug. This clearly indicates that RIF’s molecules intercalate into the lipid bilayers, as anticipated by its lipophilic nature. The bulky size of RIF is thus responsible for the increased mean diameter of the RIF-liposomes compared to empty. Similarly, liposomes containing INH have an average vesicle size of 14.66 μm. In case of liposomes containing both drugs were having vesicle size of 18.19 μm. Vesicle size of lyophilized liposomes in presence of cryoprotectant was remained unchanged; however liposomes without cryoprotectant resulted in increased particle size. After lyophilization, the particle size obtained initially was very high until the concentration of cryoprotectant sucrose was optimized. The surface morphology of liposomes was investigated and the SEM micrographs are shown in Figure 2. The morphology was found to be porous, continuous with uniform texture. SEM of optimized liposome of Rifampicin was found to be discontinuous with cluster having sharp edges. Combination liposome was found to be continuous, amorphous and uniform. Further, combination liposome containing sucrose was found to be smooth, uniform and continuous.
In vitro drug release studies were carried out in dialysis bag. The release pattern of both the drugs is shown in Figure 3 and 4. The liposomal dispersion of Rifampicin was found to release about $29.12 \pm 0.102\%$ of the drug at the end of 60 min. and about $70 \pm 0.224\%$ of the drug was released at the end of 7 h. Thus a slow and sustained release of RIF was observed. Similarly, in case of Isoniazid $31.79 \pm 0.032\%$ of drug was released at the end of 60 min while $8.24 \pm 1.06\%$ of drug was released at the end of 7 h. At the other hand where both the drugs were incorporated in liposome, it was observed that the release pattern was much slower for both drugs when compared to their individual liposome. In case of Isoniazid, $81.24 \pm 1.12$ to
70.12 ± 0.74% of the drug released after 7 h. The drug release rate is thus prolonged in case of liposome containing both the drugs. In vitro release study of pure drug solution of same concentration was carried out in a similar manner for both Rifampicin and Isoniazid. Within 60 minutes 37.6 ± 0.012% of pure Rifampicin was released while 92.8 ± 0.189% was released at the end of 3 h. In case of Isoniazid 43.9 ± 1.341% was released at the end of 60 minute and 94.2 ± 0.098% was released at the end of 3 h. These results are in line with earlier reports in which inclusion of higher proportion of cholesterol in liposome resulted in prolonged drug retention. It was observed during our optimization study incorporation of higher concentration of cholesterol into liposomes decrease the efflux of both drugs. However, incorporation of cholesterol above a particular concentration (40:60 of cholesterol and Soya lecithin) resulted in decreased entrapment of Isoniazid. Hence, the formulation was optimized to have maximum incorporation of both the drugs and at the same time retains maximum stability and integrity.

![Graph](image1)

OLF: optimized liposomal formulation containing rifampicin (RIF)
OLF+: optimized liposomal formulation of combined rifampicin (RIF)

![Graph](image2)

OLF: optimized liposomal formulation containing isoniazid (INH)
OLF+: optimized liposomal formulation of combined isoniazid (INH).

**Figure 3.** Release profile of pure RIF, INH and its OLF containing RIF and INH.

Lyophilization has been adopted in order to increase the stability of the formulated liposomes. The freeze drying cycle was optimized for efficient freeze drying process. Empty liposomal dispersions, freeze dried without cryoprotectant resulted in increase in the vesicle size. Use of sucrose in the ratio of 1:1 with lipid gave a free flowing powder. Thus sucrose as a cryoprotectant may work by stabilizing bilayers especially at their phase transition temperature, during the freezing and thawing. The DSC thermo grams corresponding to pure Rifampicin, Pure Isoniazid and optimized formulation containing Rifampicin, optimized formulation containing Isoniazid and optimum liposomal formulation containing both Rifampicin and Isoniazid are shown in Figure 1. The DSC curve of pure Rifampicin showed a single melting peak at 193° C and started to degrade as it melted. No Rifampicin melting peak was visible in the case of encapsulated optimized Rifampicin liposome. This might be due to amorphous state of the drug dispersed in the liposomes. Since there was no shift in lipid peak, it can be
concluded that there is no significant interaction occurring between the drug and lipid. Similarly, DSC curve of pure Isoniazid showed a single melting peak at 173 °C. A very small peak was visible in the encapsulated Isoniazid liposome. This might be due to leakage of drug from the liposomes but there is also no significant interaction because no shift in lipid peak was observed. In liposomal formulation containing both drugs, there was no drug peaks as well as there was no shift in the lipid peak. It can be concluded that both the drugs found to be compatible with the lipids used in the formulation. This is in accordance with IR spectra and thus it can be concluded that there was no significant interaction taking place between the drugs and lipids.

**Conclusion**

RIF and INH are antitubercular drugs which may be more effective, with fewer side effects when directly targeted to the infectious site. The liposomes prepared by phosphatidyl choline and cholesterol may therefore be used for sustained and direct targeting to the lung TB. The proportion of cholesterol is important factor in liposome formulation as it influences integrity, stability as well as hydrophilic drug entrapment in liposome. An application of Taguchi’s orthographic array method found to be effective in preparation of liposome as it reduces the total steps required in optimization of formulation.

**References**


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