RESEARCH ARTICLE

Design and Development of Simvastatin Loaded Liposomes

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ABSTRACT

Liposomes containing simvastatin were the focus of recent research. A process called thin film hydration was used to create simvastatin (SVN) liposomes. Method optimization was accomplished with the help of the BBD model. Drug content, vesicle size, surface shape, and *in-vitro* drug release were evaluated in the generated liposomes. There was no liposome that did not meet the criteria for appropriate drug content and mean vesicle size. Liposomes that did not combine and had a spherical or nearly spherical shape were seen in the surface morphology. The produced liposomes have demonstrated significant drug release. Liposomes loaded with SVN were successfully manufactured and tested.

Keywords: Simvastatin, *In-vitro* drug release, %Drug content, Liposomes

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INTRODUCTION

After intravenous administration, liposomal medications often demonstrate enhanced ADME and dynamics, which can widen the therapeutic frame, enhance efficacy, and mitigate toxicities associated with encapsulated drugs.[1,2] Our previous research has shown that incorporating oils into liposomal membranes, alongside conventional components like phospholipids and cholesterol, offers several advantages.[3,4] The aim of current study was to develop the efficacy of liposomal formulations of simvastatin. Simvastatin is a type of medication used to lower cholesterol levels, typically administered alongside a healthy diet, regular exercise, and weight loss efforts.[5,6] It is also prescribed to reduce the risk of cardiovascular complications in high-risk individuals.[7,8] Drug loading, average vesicle size, and *in-vitro* drug release kinetics were the main criteria used to evaluate the produced liposomes.[9,10]

MATERIALS AND METHODS

We purchased the simvastatin (SVN) from Sigma Aldrich in Mumbai. Cholesterol was purchased from HiMedia Bangalore, while Lipoid GmbH of Germany supplied HSPC. The oil from soybeans was sourced from Molychem in India.[11]

Preparation and Optimization of Liposomes

We used a thin film hydration approach to generate SVN-loaded liposomes[12] (Figure 1). A round bottom flask was used to combine HSPC, cholesterol, soybean oil, and the drug in a solvent system consisting of chloroform and methanol (2:1 ratio).[13,14] The mixture was sonicated to ensure proper dissolution of the excipients. Subsequently, the solution was heated in a water bath using a rotary evaporator at 60°C until a film formed. The film was then hydrated at 70°C until it dissolved completely. The hydrated film was sonicated using a probe sonicator with alternate heating cycles. This alternating heating and sonication process was repeated five times.[15,16]

Experimental Design

The Box-Behnken design (BBD) model was utilized to optimize a process using Design-Expert 13 software. Three independent factors were identified in this multidimensional optimization procedure: HSPC (F1), cholesterol (F2), and soybean oil (F3) (Table 1). The responses measured were entrapment efficiency (R1) and particle size (R2).[17,18]

Fourier-Transform Infrared Spectroscopy

FTIR analysis was used to assess potential drug-excipient interactions using the Cary-630 Agilent technology. In summary, the IR region was scanned using pure drugs SVN, CHOL, HSPC, soybean oil, and an equimolar (1 mM) physical mixture of SVN, HSPC, CHOL, and soybean oil. Concurrently, the liposomal formulations SVN-LIPO, as well as their coated dry powders, were characterized using the same procedure and conditions.[19] The spectra were captured between wave numbers 4000 and 400 cm^{-1}.

Differential Scanning Calorimetry

Thermal properties and interactions between SVN and excipients were explored through differential scanning

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Figure 1: Formulation batches of SVN-Loaded liposomes

Table 1: In BBD, variables, responses, coded levels, and limitations

Independent variables	*High (+1)*	*Medium (0)*	*Low (-1)*
X_1- HSPC (mg)	90.68	68.01	45.34
X_2- Cholesterol (mg)	92.02	51.75	11.48
X_3- Soyabean oil (mg)	56.69	31.88	7.07
Dependent variables (Factors)			
Y_1- Drug content (%) - Maximum			
Y_2- Vesicle size (nm) – Minimum			

calorimetry (DSC) analysis of both uncoated SVN and optimized coated SVN LPs (Lyophilized) using the SDT Q600 V20.9 Build 20 instrument. In order to create thermograms, samples with SVN and coated LPs were heated in a dry nitrogen environment from 0 to 500°C at a rate of 10º per minute. During the analysis, an empty metal pan was used as a reference.[20]

X-ray Diffraction

X-ray diffraction (XRD) based physical examination was carried out to examine possible drug-carrier interactions. Diffraction patterns of both uncoated SVN and optimal coated SVN lyophilized (LPs) were captured by means of an X-ray diffractometer (Bruker D8 Advance) equipped with Cu-Kα radiation (λ = 1.54 Å) operating at 40 kV and 50 mA. The diffraction angle (2θ) was scanned from 5 to 60º with raises of 0.02º at a rate of 1 s/step.[21]

Coating of SVN-loaded Liposomes

The necessary quantity of rhamnolipid for SVN was calculated and liquefied in 1-mL of methanol. Solutions were heated on a water bath at 60°C until a film formed. Subsequently, purified liposomes of each SVN were added to the respective films. The solutions were then heated in a water bath at 70°C using a hand-shaking method to ensure proper coating of the formulation.

Lyophilization of Coated Liposomes

During the lyophilization process, the lyophilizer was initially frozen for 1-hour, followed by a 20-minute warm-up period. Subsequently, a frozen sample containing vials was placed in the lyophilizer. For primary drying, a vacuum of 1.0 mbar was set for 2 hours. After the primary drying phase, the vacuum was adjusted to 0.1 mbar for the remainder of the primary drying process. Once primary drying was completed, the vacuum was set to 0.001 mbar for secondary drying. Freeze drying of the coated formulations of simvastatin-loaded liposomes was conducted to obtain dry powder.

Drug content and Drug Entrapment Efficiency Determination

A UV-visible spectrophotometer (Agilent 1800) was used to evaluate the drug entrapment efficiency (DEE) and drug loading capacity (% DLC) of the LPs that were generated. As a whole, 0.1 mL of SVN-loaded LPs was mixed with 10 mL of methanol spun in a centrifuge, and the absorbance of the resulting supernatant was recorded at 235 nm.

Mean Particle Size and Zeta Potential

Produced LPs were centrifuged for 10 minutes at 10,000 rpm. Then, 0.1 mL of supernatant was collected from the centrifuged liposomes and diluted up to 10 mL using distilled water. The zeta potential and mean particle size of the formed LPs were measured using the Malvern ZS Zetasizer (Ver. 6.20, Malvern Instruments Ltd.). At a temperature of 20°C, each measurement was repeated three times.

In-vitro Drug Release Study

In-vitro dissolution release study of SVN was conducted using the dialysis bag technique and pure API solutions. A USP type II (paddle) device was employed for dissolution. Initially, 100 mL of dissolving liquid containing phosphate buffer (pH 7.4) was added to the vessel. To provide more detail, dialysis tubes with a molecular weight cutoff of 12000 were filled with SVN (5 mg) and SVN-loaded liposomes (equivalent to 5 mg). These dialysis bags were securely tied to paddles and immersed in vessels containing the dissolution medium. The test environment was maintained at 37°C throughout the experiment, with the paddle's rotation speed set to 100 rpm.

About 3 mL of the release medium was removed and replaced with a volume of new buffer medium at predetermined intervals (0.5, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hours). The next step was to use a 235 nm UV-spectrophotometer to examine each sample. Every sample went through this procedure three times. The proportion of total drug release was calculated by plotting the release behavior against time.

Stability Study of Liposomes

For a duration of one month, liposomal formulations were kept at temperatures ranging from 2 to 8°C and at room temperature. A cryoprotectant of 5% sucrose was included in these formulations. After 15 and 30 days of storage, the formulations were tested using UV-visible spectroscopic analysis for entrapment efficiency (EE) and drug loading efficiency (DLE).

The supernatant was obtained after centrifuging 1-mL of the formulation to evaluate the EE and DLE. Next, 1 mL of methanol was added to the supernatant, and then it was sonicated for 10 minutes to make sure it was completely dissolved. Using ultraviolet-visible spectroscopy, we found that the supernatant contained SVN.[22,23]

RESULTS AND DISCUSSION

FTIR Analysis

To explore possible drug-excipient interactions amongst SVN, cholesterol, soybean oil, di-rhamnolipid, and HSPC, infrared spectroscopy investigations were carried out (Figure 2, and Table 2). None of the medicine's characteristic peaks were missing from the data, suggesting that the drug and excipients did not interact chemically. It appears that the medication remains stable within its liposomal structure.

Table 2: FTIR values of formulations

IR values of functional groups of SVN				
Sr. No.	*Functional group*	*Peak (cm^{-1}) (Standard)*	*Peak (cm^{-1}) (Observed)*	*Indication (Vibrations)*
1	O-H	3500–3200	3284	Stretching
2.	N-H	3000–2850	2923	Stretching
3.	P=O	1275–1000	1260	Bending
4.	C-H	2000–1650	1655	Stretching
IR values of functional groups of SVN-loaded LPs				
1.	O-H	3500–3200	3282	Stretching
2.	N-H	3000–2850	2919	Stretching
3.	C=O	1750–1735	1741	Stretching
4.	P=O	1275–1000	1039	Bending

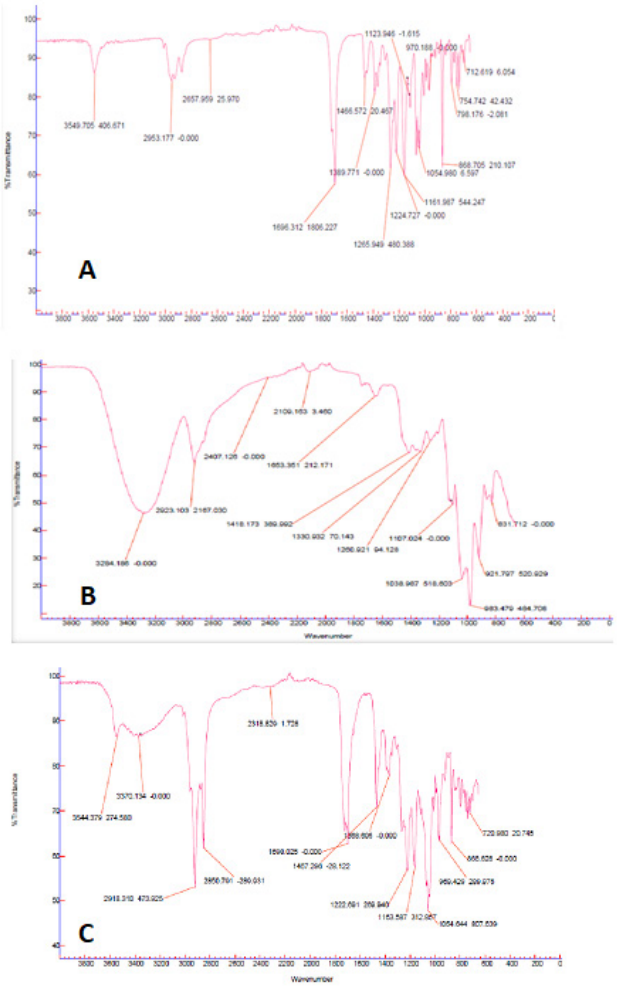

Figure 2: FTIR of A) SVN, B) Physical mixture & C) SVN loaded LPs

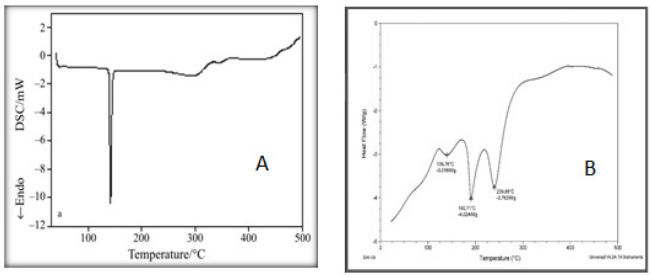

Figure 3: DSC of A) SVN & B) SVN-loaded LPs

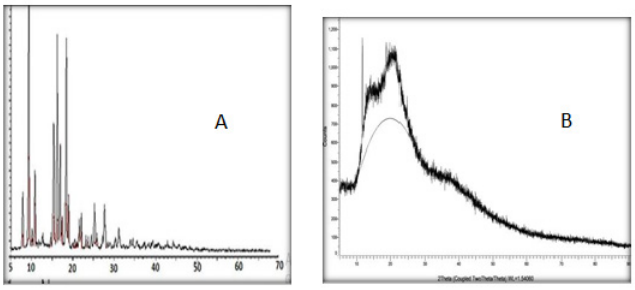

Figure 4: XRD of A) SVN & B) SVN-loaded LPs

DSC Analysis

Figure 3 displays the DSC thermograms of pure SVN alongside coated powders under a dry, inert nitrogen atmosphere. In the DSC thermogram, a peak corresponding to SVN is observed at 135 to 138°C. Additionally, an endothermic peak at 139.75°C is evident in the DSC thermogram of SVN and formulation excipients. Interestingly, the melting temperature peak of SVN decreased when excipients were enclosed in coated SVN LPs, indicating a disorganized crystallinity of molecular distribution within the polymer matrix.

XRD Analysis

Figure 4 presents the XRD patterns of standard SVN and coated lyophilized SVN. In the XRD pattern of plain SVN, distinct high-energy diffraction peaks are observed, indicating its crystalline behavior. However, the XRD patterns of coated lyophilized SVN-LPs show typical high-energy diffraction patterns, suggesting that the formulation is amorphous in nature.

Experimental design

The liposomal formulation's properties were optimized using a three-factor, three-level Box-Behnken design (BBD). For this analysis, we used the three variables. The thin film hydration approach was used to make seventeen preparation batches of SVN-liposomes. Then, in order to do statistical analysis, the gathered data on response variables were reviewed for important information. The following equation represents the derived quadratic polynomial model from the variables and responses:

$$Y= \beta0 + \beta1A+\beta2B+\beta3C+\beta4AB+\beta5AC+\beta6BC+\beta7A^2+\beta8B^2+\beta9C^2$$

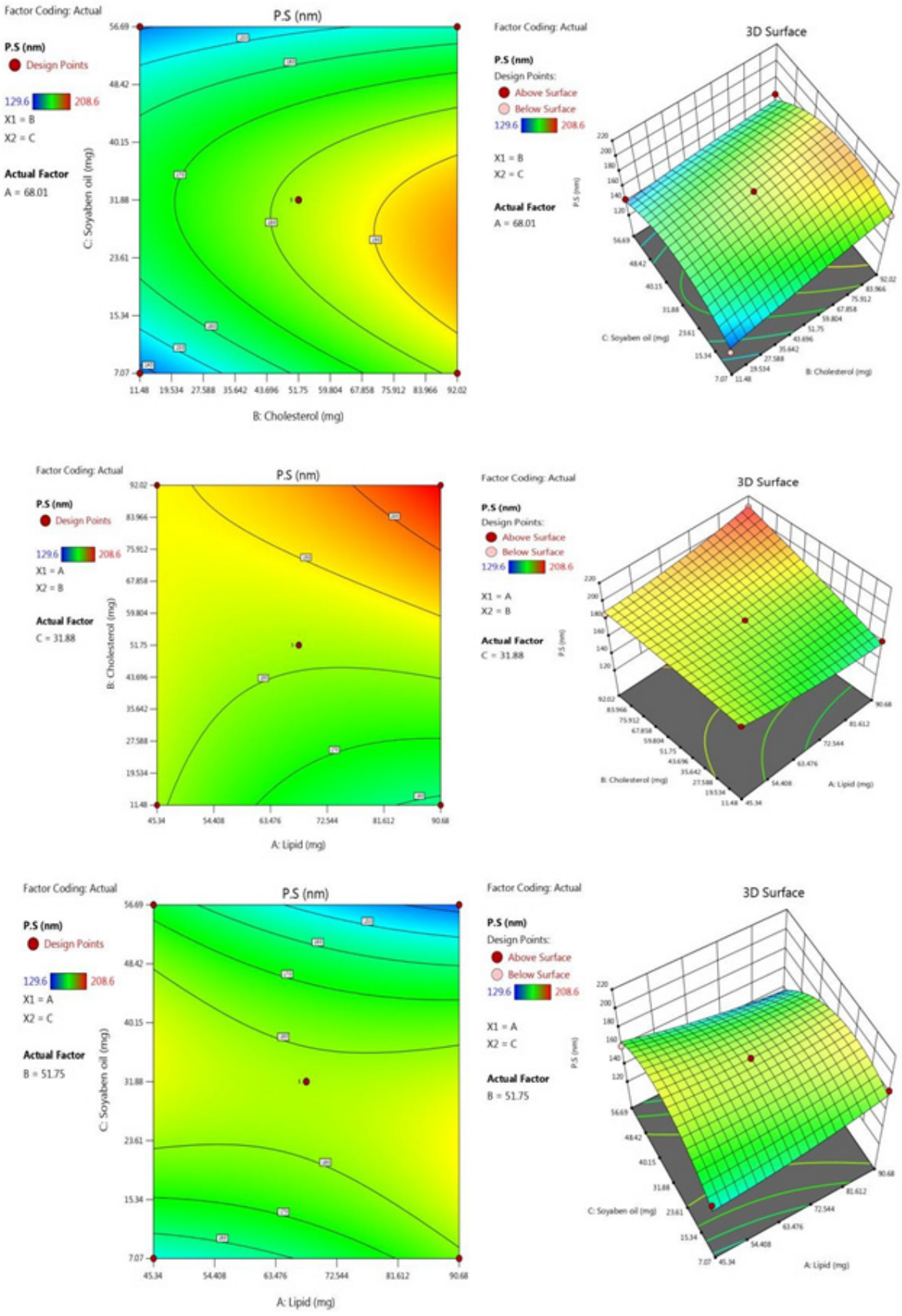

Figure 5: 2D & 3D plots showing the effect of AB, AC & BC on the particle size (Y1) of SVN

Where Y represents measured response variables (e.g., particle size, %entrapment efficiency) associated with each combination of different factor levels, as observed, the independent variables are denoted by the codes A, B, and C.

The quadratic model was found to be significant ($p < 0.05$) for the design according to the ANOVA results. The produced polynomials were considerably well-fit at $p < 0.05$, as evidenced by the corrected R^2 values of 0.9449 for particle size and 0.9119 for %entrapment efficiency.

The effective independent variables for each response were observed and determined using two-dimensional (2D) and three-dimensional (3D) graphs that were generated after the analysis of Design of Experiment (DoE) data. By analyzing the developed formulations, these graphs shed light on the impact of each variable on each response. Figures show the results of the meticulous examination that was carried out in order to determine the direct effects and interactions between variables. This information was crucial for the experimental model selection process.

Influence of Input Variables

This regression coefficient equation expresses the total influence of different factors on particle size (PS). The magnitude of the independent variables' synergistic and antagonistic impacts on the response are represented by the positive and negative signs of the regression coefficients in the equation, respectively. The 2D and 3D response surfaces showing the input variable effects on PS are shown in Figure 5. The chosen variables were shown to have a substantial impact on PS.

A significant connection between experimental and anticipated findings was indicated by the quadratic model for PS, which had a *p-value* less than 0.05 and a difference of less than 0.2 between the adjusted R^2 (0.9119) and predicted R^2 (0.7410). Furthermore, there were sufficient signals to traverse the design space, as determined by the signal-to-noise ratio (14.251 > 4), which is an indicator of sufficient precision.

Based on the response graph for PS, it appears that a higher lipid (HSPC):cholesterol ratio causes PS to rise, whereas lower ratios of CHOL:soybean oil and Lipid:soybean oil show declining PS behavior.

$$PS = +182.40i + 0.6625A + 14.71B - 6.63C + 10.85AB - 13.83AC - 7.58BC + 3.67A^2 - 1.53B^2 - 27.95C^2$$

In the proposed quadratic model, the impact of certain parameters on %entrapment efficiency (%EE) is summarized by the polynomial equation. The model was statistically significant, with a high F-value of 14.33, suggesting a low margin of error. Its agreement is further supported by the model's strong R^2 score (0.9573). The selected model is suitable for further exploration of the design space because the difference between the anticipated R^2 (0.8874) and modified R^2 (0.9449) is very small, less than 0.2.

Figure 6 shows contour plots for %EE in two and three dimensions. A high lipid:cholesterol ratio can be reached with a low %EE, as seen clearly in the graphs. The same holds true for soybean oil: lipid and CHOL: soybean oil ratios; low % EE is preferred. Based on these findings, it appears that these factors impact the %EE response.

$$\%EE = +32.20i + 1.54A + 4.36B + 4.69C - 1.91AB - 4.59AC - 1.30BC + 10.84A^2 + 8.25B^2 + 4.05C^2$$

Coating of SVN Liposomes & Lyophilization

The coating of SVN-loaded and blank liposomes was accomplished using di-rhamnolipid. Di-rhamnolipid, a microbial biosurfactant, was employed as a coating agent to enhance the microbial activity of the drug (Figures 7 and 8).

Table 3: Design matrix of SVN-loaded liposomes as per Box-Behnken design

Batches	*Factor 1 lipid (mg)*	*Factor 2 cholesterol (mg)*	*Factor 3 soyabean oil (mg)*	*Response 1 %EE (%)*	*Response 2 particle size (nm)*
1.	45.34	11.48	31.88	25.7 ± 3.55	182.2 ± 4.47
2.	90.68	92.02	31.88	50.6 ± 5.06	208.6 ± 3.68
3.	68.01	51.75	31.88	39.9 ± 3.19	182.4 ± 4.72
4.	45.34	51.75	56.69	27.3 ± 3.51	161.1 ± 4.24
5.	90.68	11.48	31.88	44.3 ± 3.30	161.1 ± 3.35
6.	68.01	92.02	7.07	43.45 ± 2.96	177.8 ± 3.55
7	90.68	51.75	7.07	52.2 ± 4.31	182.8 ± 1.59
8.	68.01	92.02	56.69	52.85 ± 4.10	261.0 ± 4.93
9.	68.01	11.48	7.07	33.55 ± 2.95	129.6 ± 4.45
10.	45.34	51.75	7.07	32.2 ± 5.12	158.4 ± 3.93
11.	68.01	11.48	56.69	48.15 ± 4.28	143.2 ± 2.60
12.	90.68	51.75	56.69	52.8 ± 5.37	130.2 ± 4.11
13.	45.34	92.02	31.88	62.1 ± 2.40	186.3 ± 2.82
14.	68.01	51.75	31.88	39.9 ± 3.19	182.4 ± 4.72
15.	90.68	51.75	7.07	52.2 ± 4.31	182.8 ± 1.59
16.	68.01	51.75	31.88	39.9 ± 3.19	182.4 ± 4.72
17.	68.01	51.75	31.88	39.9 ± 3.19	182.4 ± 4.72

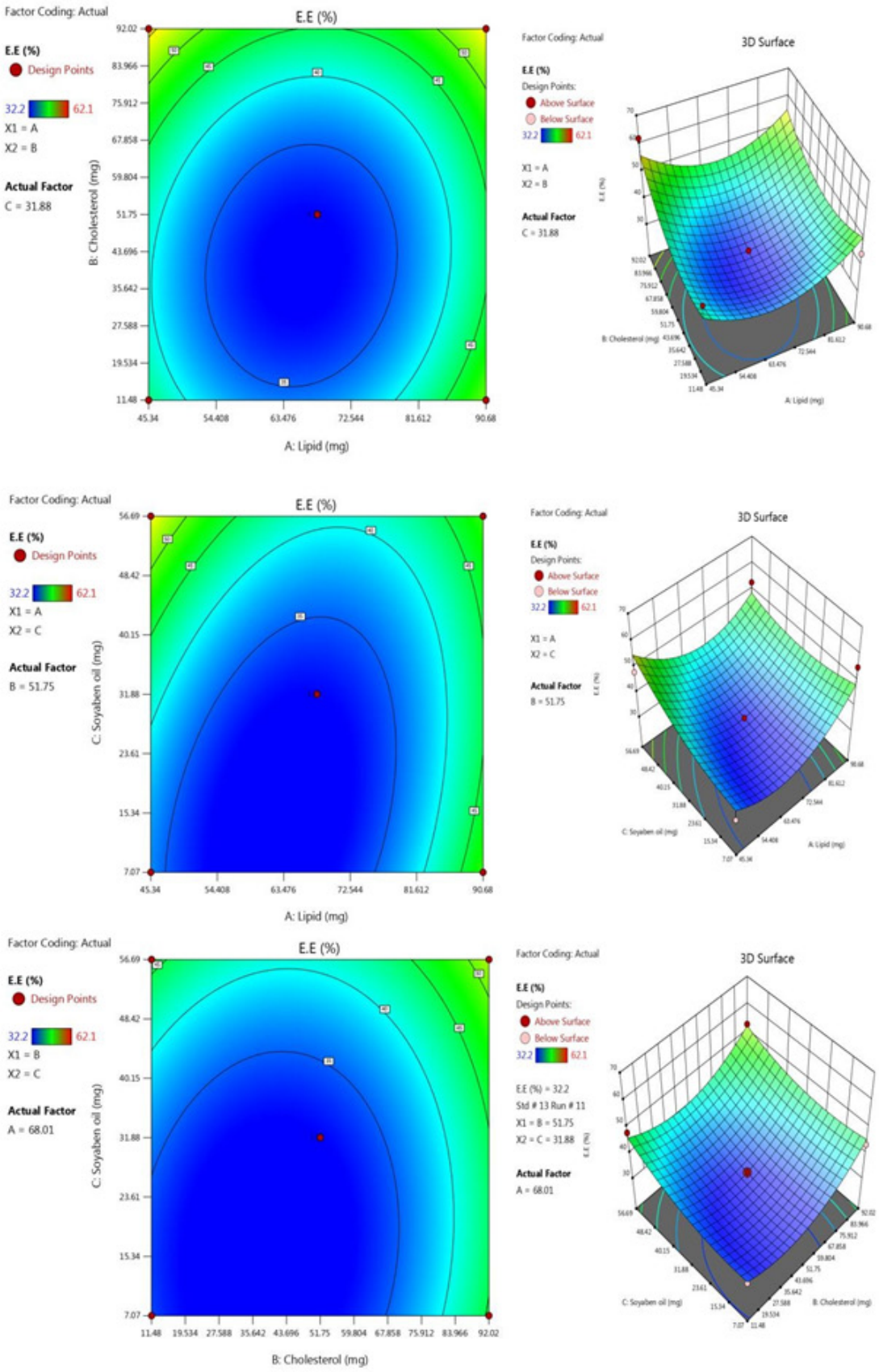

Figure 6: 2D & 3D plots showing effect of AB, AC & BC on the entrapment efficiency (Y2) of SVN

Figure 7: Coated formulations of blank liposomes, simvastatin liposomes

Figure 8: Freeze-dried coated powders of blank liposomes & simvastatin liposomes

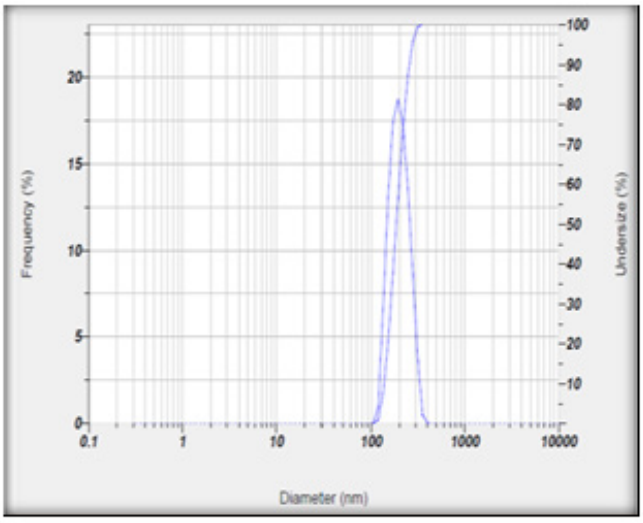

Figure 9: Particle size of optimized batch of SVN-loaded LPs

Characterization of SVN liposomes

%Entrapment efficiency

Table 3 summarizes the percentage entrapment efficiency of simvastatin into liposomes for all seventeen batches. Each batch demonstrates entrapment efficiency within a certain range. For instance, SVN batch-12 exhibited an entrapment efficiency of 52.8 ± 5.37%. It's important to note that higher entrapment efficiency indicates greater drug loading within the liposomes.

Particle size of SVN-loaded liposomes

The particle size of optimized SVN-loaded liposomes was found to be in the nanometer range, as depicted in Figure 9, Table 4.

Zeta potential of SVN-loaded liposomes

Figure 10 illustrates the zeta potential of SVN-loaded liposomes. The liposomes exhibited a negative zeta potential (-mV), indicating their anionic nature. Previous research

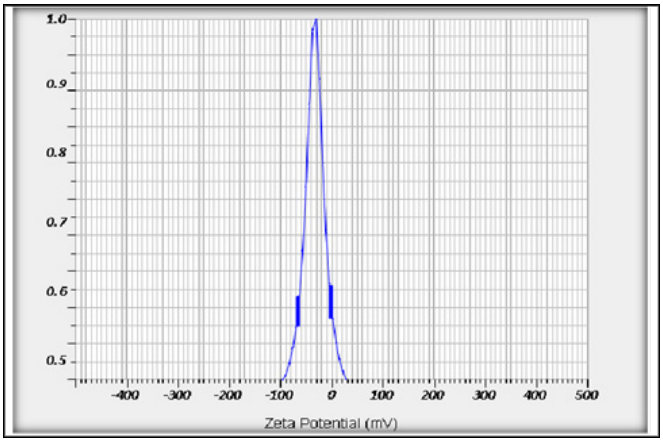

Figure 10: Zeta potential of SV-loaded LP's

Table 4: Particle size and zeta potential of optimized SVN-loaded liposomes

Batch	*Particle size (nm)*	*Zeta potential (mV)*
Optimized SVN Batch 12	130.2	-33.3

Table 5: Drug release of SVN & optimized batch of SVN

Time	*%Drug release of SVN LPs*	*%Drug release of plain simvastatin*
0.5	11.0812 ± 2.39	21.6576 ± 2.97
1	31.3604 ± 1.34	43.7079 ± 4.71
2	36.0974 ± 3.50	50.9607 ± 6.47
4	40.1921 ± 3.35	59.6319 ± 4.73
6	42.5132 ± 5.90	62.5977 ± 4.92
8	47.8659 ± 7.41	70.1425 ± 3.43
12	55.8582 ± 4.48	76.9768 ± 4.13
24	65.7556 ± 3.90	88.4533 ± 2.54
48	82.8743 ± 4.06	97.9613 ± 2.30

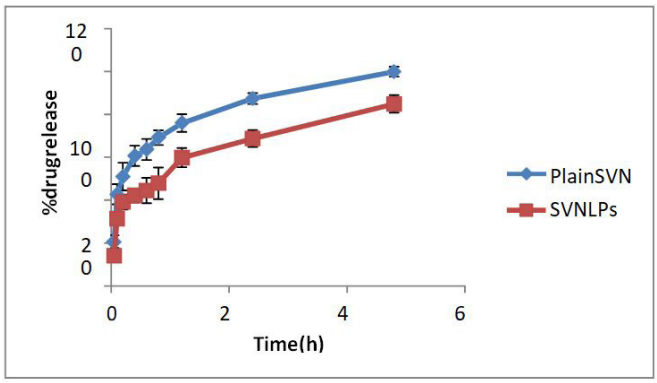

Figure 11: *In-vitro* drug release study of optimized SVN Batch 12

suggests that poly-anions possess greater bio-adhesive strength compared to poly-cations. The negative zeta potential of liposomes indicates their stability.

Drug Release Study

The drug release study is reported in Table 5, Figure 11.

Table 6: Stability study of LPs

Batch	*%EE at day 0*	*%EE after 1 month*	*%EE after 3 months*	*%EE after 6 months*
SVN 12	52.8 ± 5.37	48.4 ± 4.78	45.54 ± 5.46	40.23 ± 6.71

Stability study

The prepared SVN formulation exhibited stable results even after 1, 3, and 6 months. The stability of the formulation was confirmed, and the results are presented in Table 6. The %entrapment efficiency (%EE) of SVN was calculated using UV-visible spectroscopy at 235 nm.

CONCLUSION

The recent research focused on liposomes that contained simvastatin. Simvastatin (SVN) liposomes were created using a technique termed thin film hydration. The BBD model was used to optimize the method. The liposomes were assessed for drug content, vesicle size, surface form, and *in-vitro* drug release. All liposomes met the criterion for appropriate medication content and mean vesicle size. The surface morphology revealed the presence of liposomes that exhibited a spherical or nearly spherical form and did not aggregate. The liposomes that were created have shown a substantial release of drugs. SVN-loaded liposomes were effectively produced and evaluated.

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