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Research Article

Optimization of Protocell of Silica Nanoparticles Using 3^2 Factorial Designs

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Abstract. The purpose of the research is to carry out systemic optimization of protocells (liposomes entrapped with silica particles). Optimization was carried out using 3^2 factorial designs for the selection of the optimized protocell composition with reference to particle size distribution and zetapotential. This design was carried out to study the effect of independent variables such as molar ratio of phosphatidylcholine to cholesterol and concentration of silica nanoparticles. A total of nine formulations of protocells were prepared and analyzed using Design expert® software from Stat-Ease, Inc. (Version 8.0.4.1 trial 2010) for the selection of the optimized combination. Contour plots were constructed with independent variables like size and potential. Protocell with 7:3 ratio of phosphatidyl choline to cholesterol and 0.5 mg/ml of silica nanoparticles demonstrated better colloidal behaviors. The findings obtained from the software corresponding to independent variables demonstrated accurate means for the optimization of the pharmaceutical formulations.

KEY WORDS: central composite; Design expert®; factorial; nanoparticles; optimization; protocells.

INTRODUCTION

The liposomal drug delivery system faces certain limitations because of its poor storage stability, and burst release behavior. Protocells could provide a solution to aforesaid problems associated with liposomes. Protocells consists of porous particulate core surrounded by lipid film. The lipid bilayer of protocells improves the aerodynamics of the formulation and can be utilized for site-specific targeting of bioactives and pharmaceuticals (1–3). Pulmonary delivery of protocells provides an effective means to deliver locally active drugs (4–6). In the present study, silica nanoparticles were selected as porous particulate core for the formation of protocells. Silica nanoparticles are the nanosized colloidal carrier that provides higher surface area which improves the loading efficacy of encapsulated drugs (7). Although, the formation of silica nanoparticles is simple, involves the formation of silanol group followed by polymerization reactions which lead to the formation of siloxane bridges (8). However, optimization is necessary in the preparation of protocells, especially to improve storage stability and loading capacity of highly water-soluble biomolecules. The factorial designs aim to obtain information about the probable outcomes of cross variance with a set of independent variables (9,10). The optimization procedure based on response surface methodology (RSM) includes statistical experimental designs and analysis under a set of constrained equations. Factorial design, contour plots, and response surface methodology helps in studying the factors influencing the responses by varying them simultaneously. In the process of optimization factors like temperature, hydration time, and volume of hydration medium are kept constant while the ratio of phosphatidyl choline to cholesterol and the concentration of porous silica nanoparticles were changed to observe their effect on the development of stable formulations. The cholesterol composition plays an important role in influencing the stability and drug entrapment both in the protocells and liposomes (4). It has been found that size of the protocells affects the biopharmaceutical properties of the carrier. Thus, optimization parameter becomes a necessary step to develop a stable formulation with desired properties.

MATERIALS AND METHODS

Materials

Tetraethyl orthosilicate was gifted by Tritech Catalyst and Intermediate, Pune, India. Cholesterol, phosphatidyl choline, and bovine serum albumin were obtained from Himedia, Mumbai, India. Other chemicals used were of analytical grade.

Response Surface Design

The various equations of the response surface including linear, cubic, and quadratic (11) are as follows:

Linear

$$Y = b_0 + b_1X_1 + b_2X_2 + ... + b_kX_k \quad (1)$$

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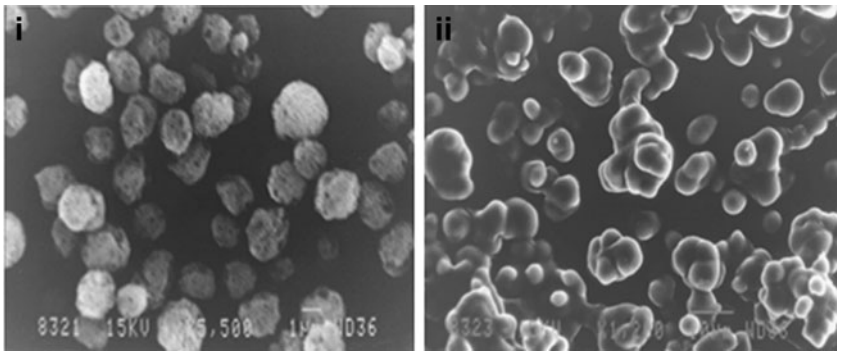

Fig. 1. Scanning electron microscopic images of samples. *i* Single plain silica, *ii* protocells

Table I. Variables and Their Levels

Variables	Low level (−1)	Medium level (0)	High level (1)
A=molar ratio of phosphatidyl choline/cholesterol ratio	6:2	7:3	8:2
B=silica concentration	0.25 mg/ml	0.5 mg/ml	0.75 mg/ml

Table II. Design of the Runs with Size and Zetapotential Obtained

Run	Factor A	Factor B	Size (nm)	Zetapotential (mV)
1	1	0	600.7	−19.09
2	0	−1	737.9	−15.45
3	−1	0	631.9	−25.46
4	1	−1	604.1	−24.38
5	−1	−1	726.6	−18.45
6	1	1	725.2	−17.98
7	0	0	664.7	−18.29
8	0	1	682.1	−24.8
9	−1	1	586.3	−58.56

Table III. Measures Derived from $(X'.X)^{-1}$ Matrix

Std	Leverage	Point type
1	0.8056	Factorial
2	0.8056	Factorial
3	0.8056	Factorial
4	0.8056	Factorial
5	0.5556	Axial
6	0.5556	Axial
7	0.5556	Axial
8	0.5556	Axial
9	0.5556	Center
Average	0.6667	

Quadratic

$$\hat{y} = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}{x_1}^2 + b_{22}{x_2}^2 + b_{33}{x_3}^2 \quad (2)$$

Cubic

$$\hat{y} = \text{quadratic model} + b_{123}x_1x_2x_3 + b_{112}{x_1}^2x_+ b_{113}{x_1}^2x_3 + b_{122}x_1{x_2}^2 + b_{133}x_1{x_3}^2 + b_{223}{x_2}^2x_3 + b_{111}{x_1}^3 + b_{222}{x_2}^3 + b_{333}{x_3}^3 \quad (3)$$

Factorial Design

The various established statistical tools are present which simplify the complex nature of the statistics involved to understand the optimizations of the pharmaceutical formulations. These tools help overcome complexities of tedious traditional methods of optimization.

Software Used: Design expert® Software from Stat-Ease, Inc. Version 8.0.4.1 Trial 2010

A flexible design structure accommodates the categoric factors, custom models, and irregular regions. The BEST optimal design results in some unusual combination of factors by trying both point exchange and the co-ordinate exchange. Optimal design is a desirability of response surface model (12).

The optimization includes the numerical optimization, graphical optimization, and the point prediction. The numerical optimization set goals for each response to generate an optimal condition. The graphical optimization set minimum and maximum limits for each response and create an overlay graph highlighting an area of operability. The point prediction predicts the response values with the confidence intervals.

The response is measured for each trial and then the model with linear, cubic, and quadratic are fitted to get the *F* test, ANOVA, and multiple regression analysis to analyze the chosen model. The fit summary evaluates the models for RSM and the mixture.

The concentration of silica nanoparticles and phosphatidyl choline/cholesterol are the major variables.

Central Composite Design

This design includes the numeric factor which can be varied over five levels: plus and minus alpha (axial points), plus and minus 1 (factorial points), and the center point. If

the categoric factors are added, the center composite design will be duplicated for every combination of the categoric factor levels. The central composite is usually centered around the optimum, more runs at the center gives a good prediction in the area. The axial replicates involve the number of times each axial run is performed. The factorial replicates involve the number of times each factorial run is to be performed. The study involves five center points, one replicate of axial point, and one replicate of factorial point.

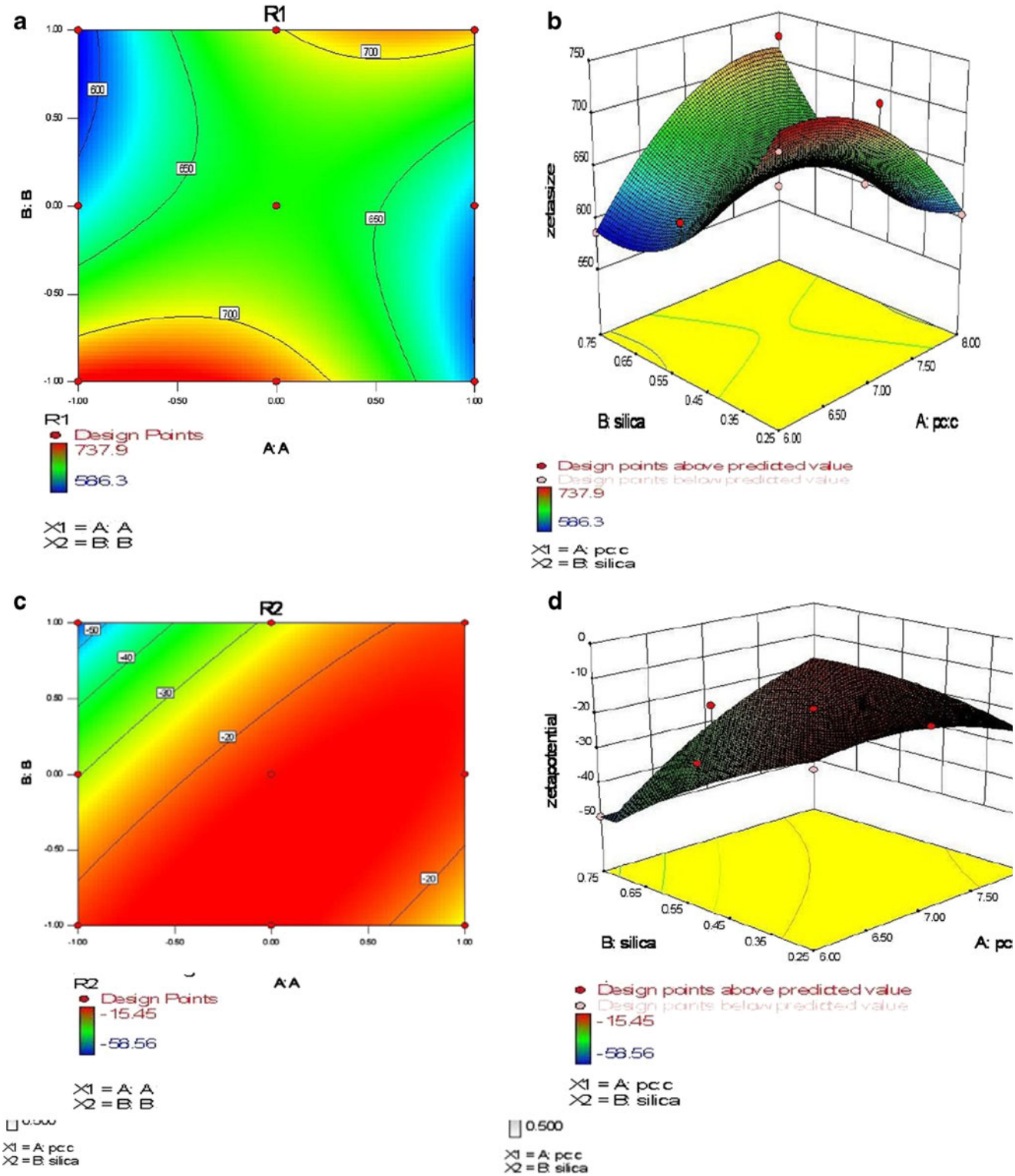

Fig. 2. Graphical evaluation of the design **a** one factor; **b** interaction with the silica (second factor); **c** contour graph representing the standard error of the design; **d** 3D graph of both factor representing the standard error of the design

Rotatable Design

In a rotatable design, the variance of the predicted values of y is a function of the distance of a point from the center of the design and is not a function of the direction, the point lies from the center. Before a study begins, little or no knowledge may exist about the region that contains the optimum response. Therefore, the experimental design matrix should not bias an investigation in any direction.

Preparation of Silica Nanoparticles (13)

Silica nanoparticles were prepared by hydrolysis of tetraethyl orthosilicate (TEOS) in ethanol in presence of ammonical solution. Ethanol (8 moles/l) and water (3 moles/l) was taken in a flask and sonicated for 10 min in bath sonicator and after 10 min a known volume (0.045 moles/l) of TEOS was added while sonicating. Twenty minute later, 28% of ammonia solution was added as a catalyst for the condensation reaction and again sonicated for an hour at ambient temperature to obtain a white turbid suspension (13). The nanoparticles were then spray dried to obtain free flowing powder. The preparation was centrifuged and washed two to three times with deionized water to remove any unreacted TEOS. Bovine serum albumin (50 μg/ml) as sample protein was loaded in the nanoparticles during sonication.

Preparation of Protocells

Different ratios of phosphatidyl choline and cholesterol were taken and dissolved in chloroform (4 mL) in RBF and rotated clockwise until a uniform film of lipids was formed. Silica nanoparticles in different concentrations were dispersed in phosphate buffer saline pH 7.4 (3 mL). This was used to hydrate the lipid film by rotating anticlockwise and was kept undisturbed for 2 h at room temperature for swelling and stored in refrigerator (1).

Surface Morphology

The surface characteristics of the silica nanoparticles and protocells were studied by scanning electron microscopy (Fig. 1). Double-sided carbon tape was affixed on aluminum stubs. The developed formulation was added onto the tape. The aluminum stubs were placed in the vacuum chamber of a scanning electron microscope (JSM 840, Jeol, Japan). The samples were observed for morphological characterization using a secondary electron detector.

Vesicle Size Determination and Zetapotential

The vesicle sizes and zetapotential of protocells were determined by particle size analyzer (Beckman Coulter–Delsa Nano C-Particle Analyzer). The sample was placed in an automated dispersion unit and subjected to particle size analysis and zetapotential 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.

Table IV. Diagnostics Case Statistics for Size

Standard order	Actual response (nm)	Predicted response (nm)	Residual
1	726.60	740.98	−14.38
2	604.10	605.35	−1.25
3	586.30	585.28	1.02
4	725.20	711.05	14.15
5	631.90	618.53	13.37
6	600.70	613.60	−12.90
7	737.90	722.27	−15.63
8	682.10	697.27	−15.17
9	647.70	665.17	−0.47

Checkpoint Analysis

A checkpoint analysis was performed to confirm the role of the derived equation and contour plots in order to predict the response.

Optimum Formula

After developing the polynomial equations for the responses size and zetapotential with the independent variables, the formulation was optimized for the response size and zetapotential.

RESULT AND DISCUSSION

The SEM images of the samples of plain silica nanoparticles and protocells were determined. Figure 1 shows the scanning electron microscopic images of the formulations.

Design

All the batches of protocells within the design were evaluated for size and zetapotential. The design obtained nine runs. Table I represents the variables and their levels and Table II represents the design.

No aliases were found for the quadratic model. Aliases were calculated on the basis of the response selection taking into account missing data points, if necessary. Degrees of freedom for evaluation include five models, five residual, and minimum three lack of fit. Thus, it is a valid lack of fit test. Variance inflation factor (VIF) measures how much the variance of the model is inflated by the lack of orthogonality

Table V. ANOVA of Models for Size of Protocells

Source	Sum of squares	Mean square	F value	P value
Model	26,856.44	5,371.29	13.11	0.0299
A	36.51	36.51	0.089	0.7848
B	937.50	937.50	2.29	0.2276
AB	178,082.49	17,082.49	41.68	0.0075
A^2	4,821.62	4,821.62	11.77	0.0415
B^2	3,978.32	3,978.32	9.71	0.0526

A molar ratio of phosphatidylcholine/cholesterol, B silica concentration in milligrams per milliliter

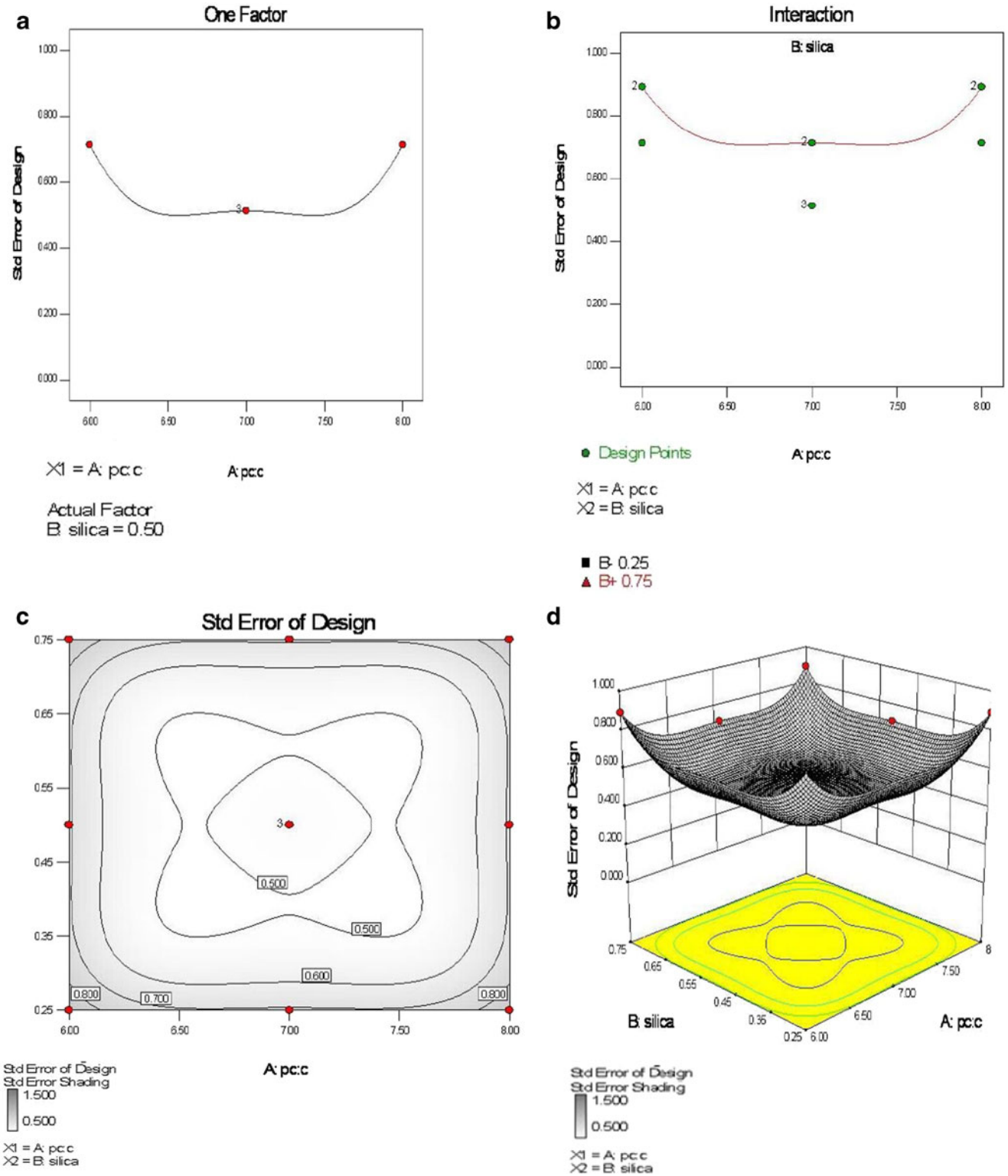

Fig. 3. Graphical analysis of size through **a** contour plot and **b** 3D. Representation of the zetapotential through **c** contour plot and **d** 3D

in the design. Ideal VIF is 1, VIF above 10 is a cause of alarm which indicates coefficients are poorly estimated due to multicollinearity. Ideal Ri^2 is 0.0. High Ri^2 means terms to be correlated with each other possibly leading to poor models. Table III represents the leverage and the point type for each run.

Graphical Evaluation of the Design

Fraction of design space plot: FDS provide assessment of merits of different designs under different parameters mis-specification (14). The plot gives the percentage of the design space volume containing a given standard error of prediction

Table VI. Diagnostic Case Statistics for Zetapotential

Standard order	Actual value (mV)	Predicted value (mV)	Residual
1	−18.45	−17.24	−1.21
2	−24.38	−26.82	2.44
3	−58.56	−54.85	−3.71
4	−17.98	−17.92	−0.062
5	−25.46	−30.39	4.93
6	−19.09	−16.71	−2.38
7	−15.45	−14.22	−1.23
8	−24.80	−29.58	3.78
9	−18.09	−15.74	−2.55

Table VII. ANOVA Results for Zetapotential of Protocells

Source	Sum of squares	Mean square	*F* value	*P* value
Model	1,316.15	263.23	10.76	0.0393
A—pc/c	280.44	280.44	11.46	0.0429
B—silica	309.03	309.03	12.63	0.0380
AB	540.80	540.80	22.11	0.0182
A^2	121.89	121.89	4.98	0.1118
B^2	64	64	2.62	0.2042

or less. This provides options for evaluating the fraction of design space as a function of the three error types:

- Mean—the standard error of the expected value or the variance of the average outcome.
- Pred—a measure of how many individual results will vary from expected.
- Diff—the difference in standard error from a number of random pairs of points picked from within the design space for power calculations that may not be readily available due to the nature of the experimental matrix.

One Factor. This evaluation graph shows the effects of changing the level of the single factor.

Interaction of Silica. This indicates the interaction of the concentration of the silica depending on the settings of the two factors.

Contour Plot. The 2D representation of the response across the selected factor.

3D Graph. The projection of the contour plot giving shape to the contour with the ability to rotate represent the 3D plot of the design.

Figure 2 shows the graphical evaluation of the design representing the standard error of the design.

DETERMINATION OF RESPONSES

The size and zetapotential obtained at the various levels of two independent variables (pc/c and silica concentration) was subjected to multiple regression to yield a final equation.

SIZE

$$\text{Size} = +665.16667 - 2.46667 \times A - 12.50000 \times B + 65.35000 \times A \times B - 49.1000 \times A^2 + 44.6000 \times B^2$$

Table IV represents the diagnostic case statistics for the response size.

The size values measured for different batches and showed 586.30 to 737.90 nm. The correlation coefficient (r^2) of the above zetasize equation was found to be 0.956. The adequate precision was found to be 9.420. The adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 9.420 indicates an adequate signal.

The model *F* value 13.11 implies the model is significant. Table V represents the ANOVA for model of size for protocells. There is only a chance of 2.99% chance that the "model *F* value" this large could occur due to noise. Values of "Prob>*F*" less than 0.0500 indicate the model terms are significant. In case of *AB*, A^2, and B^2 are significant model terms. Values greater than 0.1000 indicate the model is not significant.

Figure 3 shows the contour graph and the 3D graph of both factors in relation to the response size.

ZETAPOTENTIAL

$$\text{Zetapotential} = -157.74222 + 6.83667 \times A - 7.176674 \times B + 11.62750 \times A \times B - 7.80667 \times A^2 - 5.65667 \times B^2$$

Table VI represents the diagnostic case statistics for the response zetapotential.

The zetapotential ranges from −15.45 to −58.56. The correlation coefficient (r^2) of the above zetapotential was found to be 0.9472. The adequate precision of the model is 8.564. The adequate precision is a measurement of the signal to noise ratio. A greater than 4 is desirable, ratio of 10.060 indicates an adequate signal.

The model *F* value of 10.76 implies model to be quite significant. Table VII represents the ANOVA for model of zetapotential for protocells. There is only 3.93% chance that the "model *F* value" this large could occur due to noise.

Table VIII. Confirmation of Optimum Values of Size and Zetapotential

Factor	Name	Level	Response	Prediction	Standard deviation	SE (n=1)	95% PI[a] low	95% PI high
A	PC/C	7:3	Size	665.167 nm	20.24	25.24	584.815 nm	745.518 nm
B	Silica	0.50 mg/ml	Zetapotential	−15.74 mV	4.945	6.168	−35.373 mV	−3.8888 mV

[a] Prediction interval

Values of Prob>*F* less than 0.0500 indicate the model to be significant. In case of *A*, *B*, and *AB* are significant model terms as the value greater than 0.100 indicate model to be significant.

Figure 3 shows the contour graph and the 3D graph of both factors in relation to the response zetapotential.

OPTIMUM FORMULA

The optimum responses were determined from the study for the optimum size and zetapotential. Table VIII represents the confirmation of optimum values of size and zetapotential. The polynomial equations and the contour graphs show that the ratio of 7:3 of cholesterol to phosphotidyl choline is an optimum ratio for the development of the protocells with silica nanoparticle concentration of about 0.5 mg/ml showed optimum values of size and zetapotential.

CONCLUSION

Optimization of a protocells formulation is a complex process that requires one to consider a large number of variables and their interactions with each other. The present study conclusively demonstrates the use of a central composite design in optimization of proniosome formulations. The derived equations and contour plots predict the values of selected independent variables for preparation of optimum protocells formulations with desired properties. This software can be successfully utilized for optimization of various formulations within a given set of independent variables. Moreover, the utilization of software solution in the optimization process significantly reduced the requirement of the chemicals and save time.

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