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Research Article

Formulation of Dacarbazine-Loaded Cubosomes—Part I: Influence of Formulation Variables

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Abstract. The purpose of this study was to investigate the combined influence of three-level, three-factor variables on the formulation of dacarbazine (a water-soluble drug) loaded cubosomes. Box–Behnken design was used to obtain a second-order polynomial equation with interaction terms to predict response values. In this study, the selected and coded variables X_1, X_2, and X_3 representing the amount of monoolein, polymer, and drug as the independent variables, respectively. Fifteen runs of experiments were conducted, and the particle size (Y_1) and encapsulation efficiency (Y_2) were evaluated as dependent variables. We performed multiple regression to establish a full-model second-order polynomial equation relating independent and dependent variables. A second-order polynomial regression model was constructed for Y_1 and confirmed by performing checkpoint analysis. The optimization process and Pareto charts were obtained automatically, and they predicted the levels of independent coded variables X_1, X_2, and X_3 (−1, 0.53485, and −1, respectively) and minimized Y_1 while maximizing Y_2. These corresponded to a cubosome formulation made from 100 mg of monoolein, 107 mg of polymer, and 2 mg with average diameter of 104.7 nm and an encapsulation efficiency of 6.9%. The Box–Behnken design proved to be a useful tool to optimize the particle size of these drug-loaded cubosomes. For encapsulation efficiency (Y_2), further studies are needed to identify appropriate regression model.

KEY WORDS: Box–Behnken design; cubosomes; dacarbazine; formulation variables.

INTRODUCTION

Dacarbazine (DTIC), a water soluble drug, is currently used as a first line chemotherapy medication against melanoma (1,2). However, current therapies are not ideal. For example, the reference drug in melanoma therapy, dacarbazine, is potent (3), but it has some serious side effects. Firstly, it is normally administered intravenously, which is painful and usually not patient compliant. Secondly, the absorption of dacarbazine is generally erratic, slow, and incomplete. Thirdly, the drug is light sensitive and unstable. One promising strategy to overcome these limitations is to encapsulate this drug using nanocarriers or nanoparticulate systems intended for controlled drug delivery. In recent years, cubosomes (cubosome dispersions) entered the drug nanocarrier library as a novel member due to their great potential as an alternative drug delivery system relative to liposome. Cubosomes, especially made of binary systems, monoolein–water (4), are one of the most studied binary systems. These are aqueous surfactant systems that can self-assemble into thermodynamically stable bicontinuous cubic liquid crystalline phases. They are viscous isotropic and have a large internal surface area (~400 m^2/g) (5). Cubosomes are capable of loading lipophilic, hydrophilic, and amphiphilic drugs. Because of the three-dimensional nanostructure with hydrophobic and hydrophilic domains, cubic liquid crystalline phases have been applied in pharmaceutical drug delivery. The large interfacial area can provide a complex diffusion pathway for sustained release of entrapped drug molecules, whereas lipid constituents are biocompatible, bioadhesive, and digestible (6,7). Previous research on drug encapsulation within cubosomes concerned the study of somatostatin (8), insulin (9), indomethacin (10), and rifampicin (11). Cubosomes have also been investigated for different pharmaceutical applications (peptides, enzymes, antimuscarinic drugs, antibiotics, and analgesic delivery) and extensively reviewed (7,12–14). Although the properties of bioadhesion and penetration enhancement of cubosomes suggest their potential utility in skin cancer (e.g., melanoma) treatment, there is currently no formulation addressing this need. Moreover, there is emerging interest in using statistical methods to optimize pharmaceutical formulations (15–17). However, to our knowledge, such methods have seldom been used specifically for drug-loaded cubosome formulation. The present study is concerned with the first production and the

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characterization of cubosomes (using such methods as a novel nanomedicine) for dacarbazine that could eventually be used by a transdermal route to improve drug stability, efficacy, and safety.

MATERIALS AND METHODS

The glycerol monooleate RYLO MG 19 (GMO) was a gift from Danisco Cultor (Grindsted, Denmark). Poloxamer 407, Pluronic F127 (F127) was a gift from BASF Corporation (Ludwigshafen, Germany). Phosphate-buffered saline (PBS) and dacarbazine (DTIC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Chloroform was purchased from Fisher Scientific (Pittsburgh, PA, USA). All chemicals used in the study were of analytical grade and used without further purification.

Box–Behnken Experimental Design

Box–Behnken design is a class of second-order designs based on three-level incomplete factorial designs (18). This design constitutes three parts. In each part, two factors are full-leveled, while a third factor is set at zero level. The dots on the surface of a sphere are centered at the origin of the coordinate system and tangential to the midpoint of each edge of the cube (19).

Compared to the other experimental designs, Box–Behnken design has two major advantages. First of all, in this design methodology, three factors are needed, and only 12 runs plus three replicates at the center point are required, costing less time and energy. Secondly, each factor is studied and coded at three basic levels, an important feature in experimental design. Last but not least, since Box–Behnken design does not concern factors at extremely high or extremely low levels, they are a useful tool in avoiding experiments in extreme conditions, under which undesirable results might occur (19).

Hence, a Box–Behnken statistical design with three-level, three-factor method and 15 runs of experiments was selected for our study for the purpose of optimization. The experimental design was constituted of a set of points of high values, low values, and the replicated center point. The independent and dependent variables are listed in Table I. The polynomial equation obtained by Box–Behnken design software (JMP version 5.1, SAS Institute) is as follows:

$$Yi = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

Where Yi is the dependent variable, b_0 is the intercept, b_1 to b_{33} are the regression coefficients, and X_1, X_2, and X_3 were the independent variables that were selected from the preliminary experiments. In our research, the monoolein amount, polymer amount, and drug amount were chosen as critical factors because monoolein was the main matrix component of the cubosome, the polymer was used as surfactant, and dacarbazine was the drug to be encapsulated.

Preparation of Dacarbazine-Loaded Cubosomes

Drug-loaded cubosomes were prepared through an adapted coarse method (10). Briefly, for each sample, a volume of 15 ml of chloroform was used to completely dissolve GMO and Pluronic F127. All 15 runs of experiments were prepared according to the Box–Behnken design in Table II. The chloroform was allowed to evaporate under reduced pressure at 60 rpm and at a temperature of 60±2°C, leading to the formation of a thin film at the bottom of the flask. A volume of 50 ml of PBS buffer saline (pH=7.4) was used to dissolve the drug. This solution was added to the dry lipid film to form coarse dispersions. A sonicator was used to briefly mix the lipid film and water phase together, and the mixture was used to keep the coarse dispersions under hot water (80±2°C) for 15 min in a water bath. The hot mixture was transferred swiftly to a beaker in which a homogenizer (IKA ULTRA-TURRAX T-25, Staufen, Germany) was used for 1 min at the speed of 13,500 rpm to prepare uniform dispersion. Cubosomes were formed when the dispersion cooled down to room temperature gradually. Aluminum coils were used to cover the sample vials in order to protect samples from direct light. The dispersions were then used for future tests and evaluation.

Table I. Independent Variables and Their Levels in Box–Behnken Design

	Low	Medium	High
Independent variables			
X_1: monoolein amount (mg)	100	500	900
X_2: polymer amount (mg)	15	75	135
X_3: drug amount (mg)	2	10	18
Coded values	−1	0	1
Dependent variables			
Y_1=particle size (nm)			
Y_2=encapsulation efficiency (EE)			

Particle Size Determination

The particle size of cubosomes was determined through dynamic light scattering (DLS; Brookhaven Instruments Corporation, Austin, TX, USA). DLS, sometimes referred to as photon correlation spectroscopy, is a non-invasive, well-established technique for measuring the size of molecules and particles typically in the submicron region. The measurements were taken under the temperature of 25°C and a laser wavelength of 659.0 nm. The samples were vortexed before measuring the particle mean diameter, size distribution, and polydispersity of cubosomes. Based on a reference from National Institute Standard (NIST), a PI <0.05 was considered monodispersed (20).

Transmission Electron Microscopy

The samples were prepared by putting a 5 μl droplet of the cubosomes suspension onto a 300 mesh carbon-coated copper grid and letting the cubosomes settle for 3–5 min. Then, the excess fluid was removed. The air-dried samples were negatively stained in 1% uranyl acetate for 3–5 min. The samples were then viewed on a JEOL Model JEM 1400 120KV transmission electron microscope (JEOL-USA, Wil-

Table II. Box–Behnken Experimental Design of Independent Variables with Measured Responses

Run no.	X_1	X_2	X_3	Y_1	PI	Y_2 (%)
1	0	0	0	182.3	0.005	15.2
2	1	0	1	193.1	0.307	18.6
3	0	0	0	181.3	0.005	12.8
4	−1	1	0	115.3	0.187	16.3
5	1	−1	0	198.0	0.290	2.2
6	−1	−1	0	159.5	0.143	38.7
7	1	1	0	194.3	0.231	7.9
8	0	−1	−1	212.3	0.005	19.1
9	−1	0	1	110.9	0.243	14.9
10	0	1	1	227.0	0.005	21.4
11	0	1	−1	169.1	0.005	9.6
12	0	0	0	173.2	0.005	10.9
13	−1	0	−1	115.7	0.261	4.6
14	0	−1	1	219.0	0.239	20.9
15	1	0	−1	112.3	0.005	0.1

Y_1 mean diameter, *PI* polydispersity index, Y_2 percent encapsulation efficiency

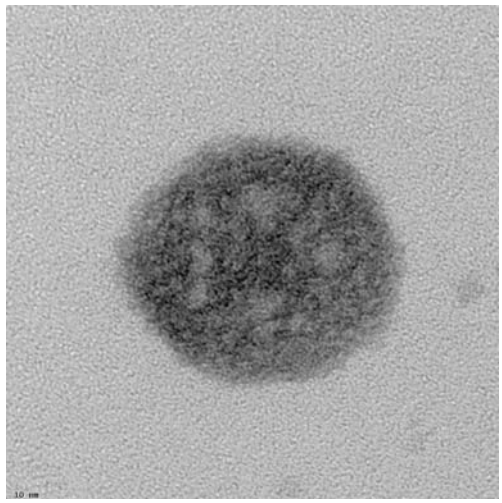

Fig. 2. Transmission electron microscopy image of cubosome (run no. 1 in Table II), which shows the cubic structure of individual cubosome. Scale bar represented 10 nm

mington, DE, USA) and photographed digitally on a Gatan axis-mount 2kx2k digital camera.

Encapsulation Efficiency

The encapsulation efficiency, or EE (Y_2), was measured at a wavelength of 330 nm with an UV spectrometer (NanoDrop Model 1000, Thermo Fisher Scientific, DE, USA). This spectrophotometer enables highly accurate UV/Vis analyses of 1 μl samples with remarkable reproducibility. The standard curve, based upon the dacarbazine concentration from 0 to 360 μg/ml, had a regression equation of $y = 0.0061x - 0.0081$ with R^2 of 0.9985. In this study, all UV measurements and calculations of Y_2 were based on this specific equipment and the standard curve. In all 15 runs of experiment, the measurement of EE was carried out with one specific kind of centrifuge tube, Amicon Ultra 3,000 MWCO (Millipore, USA). In each experiment, after the sample cooled down to room temperature, it was transferred to centrifuge tubes, Amicon Ultra 3,000 MWCO (Millipore, USA) and put to centrifuge at the speed of 1,500 rpm for 30 min. Nonencapsulated or free drug-in-solution leaked outside the sub-tubes, making it possible to measure its concentration in solution and thus allowed the deduction of the proportion of drug encapsulated in cubosomes. Preliminary studies conducted with known concentrations of drug from the calibration curve showed that this specific drug did not significantly bind to the membrane Ultra 3,000 MWCO. Moreover, the UV molar absorptivity ($\varepsilon \sim 11{,}200$ l/mol cm) did not change significantly for free and uncapsulated drug. That UV absorbance was used to compute the C_t (namely total concentration), and the UV absorbance of dacarbazine contained in filtrate after centrifuge was used to compute the C_f (namely filtrate concentration). Thus, percent EE was calculated as followed:

$$EE\% = \left[\left(C_t - C_f\right) \div C_t\right] \times 100 \qquad (2)$$

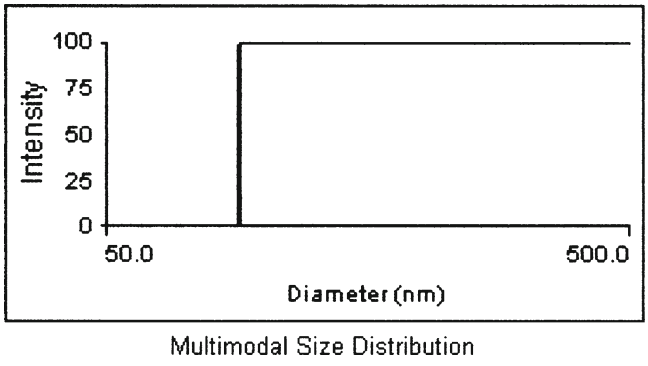

Fig. 1. DLS data image of dacarbazine-loaded cubosome of run no. 15 in Table II (the effective diameter is 112.3 nm with polydispersity of 0.005 and the elapsed time of 3 min)

Table III. Observed and Predicted Values with Residuals of the Response Y_1

Run no.	Observed Y_1	Predicted Y_1	Residuals	% Error
1	182.3	178.9	3.4	1.9
2	193.1	196.5	−3.4	−1.8
3	181.3	178.9	2.4	1.3
4	115.3	121.7	−6.4	−5.6
5	198.0	191.6	6.4	3.2
6	159.5	162.8	−3.3	−2.1
7	194.3	191.0	3.3	1.7
8	212.3	212.5	−0.2	−0.1
9	110.9	104.6	6.3	5.7
10	227.0	226.8	0.2	0.1
11	169.1	166.1	3.0	1.8
12	173.2	178.9	−5.7	−3.3
13	115.7	112.3	3.4	2.9
14	219.0	222.0	−3.0	−1.4
15	112.3	118.6	−6.3	−5.6

Table IV. Results of ANOVA of Initial and Secondary Models for Y_1 of Cubosomes Formulations

ANOVA	*Df*	SS	MS	R^2 value (Adj. R^2)	*F* value	*p* value
Regression						
A	9	22,225.18	2,469.47	0.988 (0.966)	45.16	0.0035
B	8	22,193.50	2,774.19	0.986 (0.968)	54.56	<0.0001
Residuals						
A	5	273.42 (C1)	54.69 (D1)			
B	6	305.10 (C2)	50.85			

Y_1 particle size, *A* initial (full) model, *B* secondary model
$F_{CAL} = [(C_2 - C_1)/N_{TO}]/D_1 = 0.58$, where N_{TO} is the number of terms omitted

Master Formula

Polynomial equations of the response values Y_1 and Y_2 for three-level, three-factor variables were developed after the interpretation of data (Eqs. 4 and 5, respectively). Since the polynomial equations for Y_1 fit well (R^2=0.988 and it is far more than 0.90), they were used for optimization purpose. Since the polynomial equation for Y_2 did not fit well (R^2=0.77 and it is far less than 0.90), it was not appropriate for optimization purpose. Because in nanomedicine technology, the nanoparticles with smaller size have greater potential of permeability and cellular and tissue targeting ability, for particle size (Y_1), we performed optimization toward minimizing particle size based on three levels of independent variables (X_1, X_2, and X_3).

Checkpoint Analysis

After deleting irrelevant variables and/or interactions from the initial equation, a checkpoint analysis was performed to make more sense of the analysis of secondary (reduced) equation, which was very useful in optimizing and predicting the responses. We selected three points in checkpoint analysis: two random points (−0.5, −0.5, 0) and (0.5, 0.5, 0.5) out of those 15 runs of experiments and another point (theoretically optimal point). We performed these checkpoint analyses in triplicates to ensure reproducibility. We did these experiments to check if the experimental response values obtained were approximately the same as the theoretical ones calculated by the secondary equation.

RESULTS AND DISCUSSION

Influence of Formulation Variables on Particle Size

Table II summarized the responses obtained with the Box–Behnken design for the average particle size (Y_1), the polydispersity index (PI), and the encapsulation efficiency (Y_2). The phase diagram of monolein–poloxamer system has been extensively studied (6,10). These literatures served as the basis for the selection of our factors and their level. In previous literatures, cubosomes were prepared with two distinct technologies: top-down technique and bottom-up technique (14). We used simplified top-down technique (as "coarse method" described in "Preparation of Dacarbazine-Loaded Cubosomes") because it is advantageous: less time and energy consuming and more economic. Figure 1 showed a typical size distribution of the dispersion after DLS analysis. Figure 2 also provided an electron micrograph of the cubosomes after negative staining suggesting the countours of a cubic ultrastructure in the nanosize range. These data suggested that cubosomes were indeed formed with particle size ranging from 110 to 227 nm. Similar size range of cubosome dispersions were reported by Esposito *et al.* (10). The PI of the sample varied from to 0.005 to 0.307. According to NIST standards (20), almost half of the 15 runs were monodispersed while the other half was not. It was speculated that the polydispersed formulations were made of the coexistence of cubosome with other type of vesicles as previously reported (6,21). It has been reported that in order to produce colloidally stable cubic phase dispersions, the GMO/F127 ratio should be in the range of 94/6–80/20 *w*/*w* (6). Among our runs, only run nos. 1, 2, 3, 6, 12, and 15 fitted these criteria and were all monodispersed except run nos. 2 and 6. Overall, the samples were milky emulsions in appearance, and Table III showed the observed and predicted values with residuals of response Y_1 (particle size). According to the analysis of data of 15 runs of

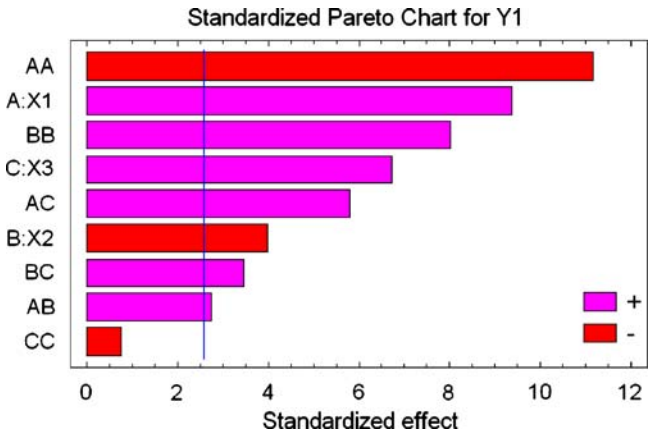

Fig. 3. Pareto chart shows the standardized effect of formulation independent variables and their interaction on Y_1 in initial (full) model. *Bars extending past the line* indicate values reaching statistical significance (α=0.05). [*A* monoolein amount, *B* polymer amount, *C* drug amount, *AA* interaction of monoolein amount and monoolein amount, *BB* interaction of polymer amount and polymer amount, *CC* interaction of drug amount and drug amount, *AB* interaction of monoolein amount and polymer amount, *AC* interaction of polymer amount and drug amount, *BC* interaction of polymer amount and drug amount]

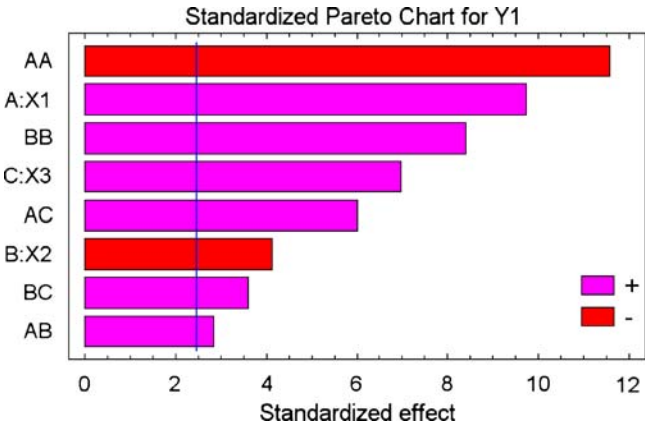

Fig. 4. Pareto chart shows the standardized effect of formulation independent variables and their interaction on Y_1 in reduced model. *Bars extending past the line* indicate values reaching statistical significance (α=0.05; same as in Fig. 3)

experiments, the initial polynomial equation (full model) for particle size (Y_1) was:

$$Y_1 = 178.93 + 24.54X_1 - 10.39X_2 + 17.58X_3 + 10.13X_1X_2 + 21.4X_1X_3 + 12.8X_2X_3 - 43.00X_1^2 + 30.85X_2^2 - 2.93X_3^2 \quad (3)$$

Where the coded factors were X_1=(natural monoolein amount−500): 400; X_2=(natural poloxamer amount−75): 60; and X_3=(natural drug amount−10): 8.

From Table IV, we could see that the correlation coefficient (R^2) value of Eq. 3 was 0.988 (Adj. R^2=0.966), indicating that this model fit the data very well. The Y_1 values in 15 runs had range from minimum value 110.9 nm to maximum value 227.0 nm. The lack-of-fit test showed that the F ratio was 2.9931 (p>0.05), which indicated that in 95% confidence level, there was no lack of fit in this model. This model and the polynomial equation satisfy the data very well. The analysis clearly stated that the Y_1 value could be explained by the variables selected for this study. In Eq. 3, the interaction terms (X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2) show how the Y_1 changes when two variables are simultaneously changed. The positive signs in coefficients (such as X_1, X_3, X_1X_2, X_1X_3, X_2X_3, and X_{22}) indicated positive effect on the Y_1, while the negative signs in coefficients for the interactions between two variables (such as X_2, X_1^2, and X_3^2) indicated the negative effect on the Y_1. These data suggested that the increase in the amount of monolein or amphiphile (monoolein and surfactant) resulted in an increase in the particle size. These results are in agreement with the work of Barauskas and colleagues (6). Moreover, the drug amount positively interacted with both amphiphile to increase particle size, suggesting an electrostatic interaction perhaps leading to more coalescence of the particle in the dispersion. For example, the drug contains amino groups that could be protonated leading to the formation of a positively charged drug in the cubosome matrix. This may lead to the modification of the charge density (zeta potential) and a reduction of the energy barrier between the particles, therefore leading to an increase in particle size or to structural transition. Electrostatic effect of additive such as oleic acid (6) and the influence of four different charged designer lipid-like peptide in tuning the curvature and stability on monoolein bilayers have been recently reported (22). The negative effect of the poloxamer amount clearly underscore its role as stabilizing agent in the formulation as it contributed to maintain the particle size toward lower range due to steric repulsion. Among the three independent variables, the coefficients whose p>0.05 were insignificant in prediction of Y_1. Two Pareto charts were constructed (Figs. 3 and 4) to compare the significance of other factors and interactions of factors before and after omission of insignificant one.

The standardized effect of the independent variables and their interaction on the dependent variable was showed in Pareto chart (Fig. 3), which indicated the main effect of the independent variables and interactions that will exert significant influence on the Y_1 value. The factors whose length passes the line (p value equals to 0.05) indicated significance on the response value. The value of that significance line in Fig. 3 was 2.58, and the value of the significance line in Fig. 4 was 2.48. The difference could be explained by the distribution change in t-curve. According to the chart in Fig. 3, X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2 contributed significantly to Y_1 value while X_3^2 did not. It is important to underscore the physical meaning of the first three terms that have been provided in Table I. The other terms are interactions between each of the previous terms. For example, the physical meaning of X_1X_2 is the concomitant effect of both the drug amount and the polymer effect on the measured response. Hence, X_3^2 omitted from the full model to obtain a reduced second-order polynomial equation (Eq. 4):

$$Y_1 = 177.13 + 24.54X_1 - 10.39X_2 + 17.58X_3 + 10.13X_1X_2 + 21.4X_1X_3 + 12.8X_2X_3 - 42.78X_1^2 + 31.07X_2^2 \quad (4)$$

From Table IV, the R^2 value in reduced equation was 0.986 (Adj. R^2=0.968). Although there was a small difference between R^2 value of the initial equation (Eq. 3) and that of the reduced equation (Eq. 4), it was statistically insignificant. The lack-of-fit test again showed that the F ratio was 2.5629

Table V. Checkpoint Experiments Comparing Measured Predicted Y_1 Value (n=3)

Run no.	X_1	X_2	X_3	Measured Y_1	Predicted Y_1	% Error
C_1	−0.5	−0.5	0.0	167.1±0.9	169.7	−1.5
C_2	0.5	0.5	0.5	204.3±1.1	201.1	1.6
C_3	−1.0	0.53485	−1.0	106.1±0.8	104.7	1.3

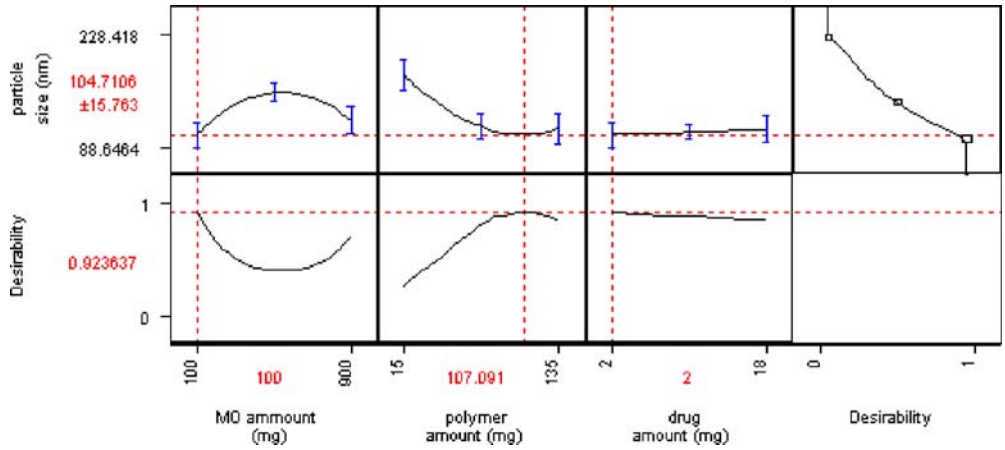

Fig. 5. Prediction and desirability plot of reduced model showing the effect of monoolein amount (X_1) and polymer amount (X_2) on the particle size of formulation part when drug amount (X_3) was set at the center point

($p>0.05$), which indicated that in 95% confidence level, there was no lack of fit in this model. To ensure the omission of insignificant terms in formulation variables, the analysis of variance for initial model and reduced model was performed with the results shown in Table IV. The calculated F (F_{CAL}) value (equals to 0.58) was less than the tabled value of F, which equals to 4.39 at $\alpha=0.05$ risk level ($Df_1=5$ and $Df_2=6$). Hence, it was concluded that omitting X_3^2 term did not significantly influence the precision of Y_1 values with 95% confidence. This indicated that the effect of monoolein and dacarbazine amount, polymer amount, and the interactions of monoolein–polymer, monoolein–drug, and polymer–drug were evident as shown in Fig. 4.

The relationship between three-level, three-factor variables and response value Y_1 in reduced model was further investigated by constructing prediction and desirability plot. In Fig. 5, it was evident that lower level of X_1 (amount of monoolein) and X_3 (drug amount) favored smaller Y_1 value of cubosomes, while the medium level of X_2 (polymer amount) favored the Y_1 value of cubosomes. These observations were also in agreement with the study of Barauskas (6).

Checkpoint Analysis

According to the reduced second-order polynomial equation (Eq. 4), two specific points (−0.5, −0.5, −0.5) and (0.5, 0.5, 0.5) and another point (−1, 0.53485, −1) were prepared and evaluated for Y_1, as shown in Table V. Results indicated that the measured Y_1 values were approximately the same as predicted. As the differences between measured Y_1 value and predicted Y_1 values were found to be insignificant ($p>0.05$) when we compared the measured values to the predicted values using Student's t test: checkpoint C1: $t=-2.89$, $Df=2$, $p=0.10$; checkpoint C2: $t=2.91$, $df=2$, $p=0.10$; checkpoint C3: $t=1.75$, $df=2$, $p=0.22$. The results of these t tests showed that none of the observed values was significantly different than the predicted values, which was good evidence that the regression equation is an accurate model of the data. Therefore, we concluded that the secondary equation fits the data satisfactorily and was valid for predicting the Y_1 values.

Master Formula

From the data analysis of the effect of independent variables on the responses, it was obvious that three-level, three-factor variables would give the optimum response values about Y_1 in formulation section. It was evident from the polynomial equation and Pareto chart (Fig. 4) that Y_1 increases with the increase in monoolein amount (X_1) to a level of 100 mg, but decreases at amounts above 100 mg. Y_1 increases with drug amount (X_3), the interaction of polymer amount (X_2) with polymer amount (X_2), the interaction of monoolein amount (X_1) with polymer amount (X_2), the interaction of monoolein amount (X_1) with drug amount (X_3), and the interaction of polymer amount (X_2) with drug amount (X_3). Y_1 decreases with the decrease in polymer amount (X_2) and the interaction of monoolein amount (X_1) with monoolein amount (X_1) as these materials constituted the bulk of the dispersed phase. Hence, to minimize particle size, the lower level of monoolein amount and drug amount was selected, and the medium level of polymer was selected. For controlled-delivery purpose based on nanomedicine concept, the optimal formulation would be the one that would reveal the lowest particle size (23). Using a computer optimization process and the interaction plot shown in Fig. 5, we selected a level of −1 for both X_1 and X_3 and a level of 0.53485 for X_2, which are as a desirability ($d=0.9236$) that is very close to the ideal value of 1. The theory behind the ideal desirability value of 1 based on desirability function for simultaneous optimization of several response variables has been popularized by Derringer and Suich (24).

Table VI. Results of ANOVA of the Initial Model for Y_2 of Cubosome Formulations

ANOVA	*Df*	SS	MS	R^2 value	*F* value	*p* value
Regression Eq. 5	9	0.0966	0.0107	0.77	1.8512	0.0677
Residuals Eq. 5	5	0.0289	0.00578			

Y_1 encapsulation efficiency

The optimal Y_1 value was 104.7 nm after computation. For confirmation purpose, three fresh samples were prepared at the optimum levels of the independent variables, and the resultant cubosome had an observed value of particle size of 106.1 nm (in Table V), which was in close agreement with the theoretical values.

Influence of Formulation Variables on Encapsulation Efficiency

In this study, we investigated the optimization for formulation variables. According to the statistical analysis, we found that the model fit Y_1 very well (R^2>0.90), while the model had a lack of fit for Y_2. The initial polynomial equation (full model) for encapsulation efficiency (Y_2) is:

$$Y_2 = 0.130 - 0.057X_1 - 0.032X_2 + 0.053X_3 + 0.070X_1X_2 + 0.020X_1X_3 + 0.025X_2X_3 - 0.024X_1^2 + 0.058X_2^2 - 0.010X_3^2 \quad (5)$$

As shown above in Table VI, R^2 value of EE model is 0.77 and is less than 0.90, which indicates that the model is not as good as the one used for particle size prediction. It could not be used to predict encapsulation efficiency due to lack of fit. Even when we performed the Box–Cox Y transformation (25) on EE, the R^2 was still less than 0.90 (data not shown).

In this case, our current model was not suitable for analyzing and predicting the drug encapsulation efficiency. In the future, experimental design and research may investigate comprehensive factors in order to construct a better model for Y_2 values.

During the process of analyzing data, we needed to omit some parameters and/or interactions of parameters to obtain optimization equations. Concerning the omission of parameters, one rule should be kept to direct the whole process. We omitted only one parameter, the interaction of drug amount with drug amount, to improve the R^2 value. By doing so, we would improve the availability of the polynominal equation without affecting the significance of other parameters or interactions of parameters. In this case, other parameters and interactions of parameters would become significant after the omission.

CONCLUSION

This study reports on the first use of a Box–Behnken design in the optimization cubosome dispersion mean diameter for the encapsulation of dacarbazine (an antimelanoma drug). The derived polynomial equations and Pareto charts proved to be satisfactory in predicting Y_1 values for the preparation of optimum cubosomes with desired particle size. The optimal formulation size could be obtained when 100 mg of monoolein, 107 mg of polymer, and 2 mg of drug as independent variables leading to the formulation of cubosomes with 104.7 nm in mean diameter and 6.9% in encapsulation efficiency. The relatively low encapsulation may be due to the rapid diffusion of the drug in the aqueous phase during the preparation and the centrifugation process because it has been reported that even lipophilic drugs were rapidly release from cubosomes after ultrafiltration (11). Although the model was not good enough to predict encapsulation efficiency in our case, the Box–Behnken design shed light on future study on fit model. Further experiments should explore a preparation condition and better prediction model for the drug encapsulation efficiency (Y_2) and study the process parameters in order to increase encapsulation efficiency. Also, further physicochemical characterization method would be needed to better elucidate the ultrastructure of the cubosome dispersion.

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