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Research Article

Statistical Design for Formulation Optimization of Hydrocortisone Butyrate-Loaded PLGA Nanoparticles

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Abstract. The aim of this investigation was to develop hydrocortisone butyrate (HB)-loaded poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles (NP) with ideal encapsulation efficiency (EE), particle size, and drug loading (DL) under emulsion solvent evaporation technique utilizing various experimental statistical design modules. Experimental designs were used to investigate specific effects of independent variables during preparation of HB-loaded PLGA NP and corresponding responses in optimizing the formulation. Plackett–Burman design for independent variables was first conducted to prescreen various formulation and process variables during the development of NP. Selected primary variables were further optimized by central composite design. This process leads to an optimum formulation with desired EE, particle size, and DL. Contour plots and response surface curves display visual diagrammatic relationships between the experimental responses and input variables. The concentration of PLGA, drug, and polyvinyl alcohol and sonication time were the critical factors influencing the responses analyzed. Optimized formulation showed EE of 90.6%, particle size of 164.3 nm, and DL of 64.35%. This study demonstrates that statistical experimental design methodology can optimize the formulation and process variables to achieve favorable responses for HB-loaded NP.

KEY WORDS: central composite design; hydrocortisone butyrate; nanoparticles; Plackett–Burman; PLGA.

INTRODUCTION

Corticosteroids (or "steroids" for short) are synthetic derivatives of natural anti-inflammatory hormones secreted by adrenal glands (1,2). These compounds can relieve inflammation in various cells to prevent release of modified chemicals that are normally involved in producing immune and allergic responses, resulting in inflammation (3). The corticosteroids in ester forms are widely applied as topical anti-inflammatory agents, e.g., hydrocortisone acetate, and hydrocortisone butyrate (HB) (4,5). Given their potent anti-inflammatory activity, these agents are considered to be therapeutic candidates in the treatment of ocular inflammation. In eye clinics, corticosteroids are widely administered by eye drops, suspension, creams, and ointment (6,7); 0.1% HB ointment showed no skin and eye irritations in rabbits (8,9). However, these formulations exhibit low bioavailability and may cause adverse effects such as glaucoma, transient stinging or burning, blurred vision, and local irritation (10). Moreover, the conventional ophthalmic formulations of steroids such as hydrocortisone and hydrocortisone acetate can only achieve short-term relief of ocular inflammation, owing to their very short residence time in the precorneal area. Therefore, steroid thereby requires frequent administration (11). Alternative drug delivery systems such as implants and intravitreal injections may overcome such disadvantages associated with conventional topical delivery systems. However, there is limited patient compliance because of surgical procedure, discomfort, and many adverse effects (12,13).

To overcome these limitations, our aim is to develop HB-loaded biodegradable nanoparticles (NP) which may provide a sustained release. Poly(D,L-lactic-co-glycolic acid) (PLGA) has been the most widely applied biodegradable NP for many years. This polymer is easy to use, biocompatible, biodegradable, and generally nontoxic lactic and glycolic acid *in vivo* (14).

PLGA NP can be formulated by emulsion solvent evaporation (*o/w*) technique (15). This well-established method involves emulsification of water-immiscible organic solvent containing PLGA polymer into drug in an aqueous surfactant aqueous solution with steroid under sonication. Removal of organic solvent under vacuum generates NP. During this manufacturing process, many parameters such as the concentration of polymer, drug, surfactant, duration and intensity of sonication, and volume of aqueous phase must be optimized to obtain HB-loaded NP. It is, therefore, essential to observe the effects of these preparation variables and their potential interactions among factors, i.e., physicochemical properties. However, it is costly and time consuming to assess the impact of a large number of formulation factors individually. Design of experiments can be successfully implemented to minimize the total number of experiments and identify the dominant

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variable effects and interactions (16,17). A screening design such as Plackett–Burman can initially be selected to determine the most important parameters among a number of independent variables with a limited number of experiments (18,19). This statistical method, however, neglects the interactions among the factors (20).

Central composite design (CCD) is a well-known method involving mathematical modeling and statistical analysis to evaluate critical variables responsible for optimization of product quality (21). It consists of imbedded factorial design, star points for estimation of curvature, and center points for evaluation of experimental reproducibility (22). It can significantly reduce the number of empirical experiments in determining mathematical trends without sacrificing final product quality. The method generates a specific mathematical model that provides information on how the response is influenced by several variables, which allow the prediction of optimum level of variable factors required for a given response.

Therefore, the objective of the present study is to optimize independent variables in order to achieve desired encapsulation efficiency (EE), particle size, and drug loading (DL) for the HB-loaded NP. The objective is also to employ application of statistical data to appropriately modify methodologies in research and/or practice and to incorporate a practical predictive statistical process including experimental design, parameter estimation, model predictive control, and statistical analyses in the production and optimization of formulations.

MATERIALS AND METHODS

Materials

Hydrocortisone 17-butyrate was purchased from Sigma-Aldrich Co. (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade acetonitrile and dichloromethane were purchased from Fisher Scientific Co. (Fairlawn, NJ). PLGA copolymer with a monomer ration of 75:25 (Mw 66,000–107,000 Da) and polyvinyl alcohol (PVA; Mw 31,000–50,000 Da) were obtained from Sigma-Aldrich Co. Double-distilled deionized (DDI) water was used throughout in the preparation of formulations and buffers. Ultrapure water was prepared using a Milli-Q® water purification system (Millipore Co., Billerica, USA) and filtered (0.22 μm) before use in mobile phases. All other chemicals in the study were of analytical reagent grade.

Methods

Preparation of NP

PLGA NP containing HB were prepared by emulsion solvent evaporation method (23). Briefly, various amounts of PLGA and HB were dissolved in dichloromethane (5 ml) to form the organic phase. It was then slowly mixed with an aqueous solution containing PVA and an oil/water-type emulsion was formed upon sonication over an ice bath using a Fisher Scientific 100 ultrasonic probe. Organic solvent was removed by stirring at room temperature for 6 h and then evaporated under a vacuum for 30 min to ensure complete removal of organic solvents. To remove the unentrapped drug and PVA, NP suspension was washed with DDI water followed by centrifugation at 5,000×*g* (22,000 rpm) for 1 h. This procedure was repeated for three times. Resulting NP suspension was freeze-dried over 48 h under a freeze-drier system to obtain NP in powder form.

Characterization of Physicochemical Properties of NP

1. *Entrapment efficiency and DL*

For measuring drug entrapment in NP, 1 mg of freeze-dried material was dissolved in 2 ml of dichloromethane and vortexed for 1 min to completely solubilize the NP. Subsequently, the mixture was dried under inert atmosphere (nitrogen gas) and dissolved in 500 μL of acetonitrile/water (60:40) by vortexing. After centrifugation at 12,000 rpm for 10 min, the supernatant was aspirated for analysis of drug concentration by HPLC. Entrapment efficiency and DL were calculated with Eqs. 1 and 2. The measurements were performed in triplicate.

$$\text{Entrapment efficiency}(\%) = \frac{\text{Weight of drug in nanoparticles}}{\text{Initial weight of feeding drug}} \times 100 \quad (1)$$

$$\text{Drug loading}(\%) = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of nanoparticles}} \times 100 \quad (2)$$

2. *Particle size and polydispersity by dynamic light scattering*

The average particle size distribution and polydispersity index (PDI) of freeze-dried NP were characterized by dynamic light scattering analyzer (Brookhaven Zeta Plus instrument, Holsville, NY). Analyzers were performed in triplicate.

HPLC Analytical Method

Samples collected were analyzed by HPLC system (Waters 600 pump, Waters, Milford, MA) equipped with a fluorescence detector (HP1100, Hewlett Packard, Waldbronn, Germany). The analysis condition included a Kinetex 2.6 μm PFP 100 A column (50×2.1 mm ID; Torrance, CA, USA); the mobile phase comprised a mixture of water (pH 6.5) and acetonitrile (62:38, *v*/*v*) and flow rate of 0.6 ml/min at 25°C. The wavelength of UV detection was 240 nm with a sensitivity 0.0010 AUFS. Fifty microliters of sample volume was injected. The retention time t_R was found to be 7.78 min.

Experimental Design

In this study, the effects of formulation and process parameters on various response properties were investigated by experimental designs (24). The independent variables such as polymers, drug, and surfactant were examined. The concentration of polymer, drug, and surfactant, the duration and intensity

Table I. The Initial Input Factor Levels and Experimental Responses in Screening Design Plackett–Burman

	Input factor levels						Experimental response			
	1	2	3	4	5	6				
Run no.	PLGA (mg/ml)	Drug (mg/ml)	Water volume (ml)	PVA (%)	Sonication power (W)	Sonication time (min)	EE (%)	Particle size (nm)	DL (%; *w/w*)	PDI
1	37.5	3	6	2	45	8	89.4	191	7.15	0.005
2	37.5	9	6	1	75	4	83.8	208.6	20.11	0.021
3	37.5	9	14	1	75	8	81.5	200.3	19.56	0.036
4	12.5	9	14	2	45	4	58.6	192.1	42.19	0.007
5	37.5	3	14	2	45	4	96.2	192.9	7.70	0.082
6	12.5	9	6	2	75	8	49.7	187.9	35.78	0.130
7	12.5	3	14	1	75	8	53.4	198.4	12.82	0.119
8	12.5	3	6	1	45	4	58.8	209.3	14.11	0.104

PLGA poly(D,L-lactic-co-glycolic acid), *PVA* polyvinyl alcohol, *EE* encapsulation efficiency, *DL* drug loading, *PDI* polydispersity index

of sonication, and the volume of aqueous phase were varied. The upper and lower limits for individual factors, generating stable formulation under solvent evaporation technique, were identified in the preliminary studies (not shown here). The response considered included EE, particle size, and DL.

Plackett–Burman is a type of screening design initially conducted to identify the significant main factors rather than interaction parameters among the 6 factors (25). In this prescreening experimental design, a total of eight experiments were carried out and structured for 6 factors with 2 levels. The experimental design with input and output values is shown in Table I. Based on Plackett–Burman design, 4 critical factors were selected as most important independent variables. In the second step, these 4 critical factors were implemented in CCD to predict the optimum level and to achieve desirable responses including EE, particle size, and DL. In this experimental design, 27 different runs were performed to determine the model coefficients. The first 16 runs are presented in Table II involving 2^4 full factorial design, where 4 corresponds to the number of critical factors, and 2 to the number of levels for each factor. Eight star points

Table II. Central Composite Design for Four Factors at Three Different Levels

	Input factorial levels					Experimental response			
Run no.	X_A/PLGA (mg/ml)	X_B/drug (mg/ml)	X_C/PVA (%)	X_D/sonication time (min)	Experimental design	EE (%)	Particle size (nm)	DL (%, *w/w*)	PDI
1	12.5	3	1	4	2^4 factorial design	96.61	212.7	23.19	0.072
2	12.5	3	1	8		86.82	151.8	20.84	0.008
3	12.5	3	2	4		96.61	208.2	23.19	0.043
4	12.5	3	2	8		86.83	141.8	20.84	0.006
5	12.5	9	1	4		95.36	209.1	68.66	0.011
6	12.5	9	1	8		85.55	146.1	61.60	0.005
7	12.5	9	2	4		95.56	192.8	68.80	0.005
8	12.5	9	2	8		83.45	142.4	60.08	0.009
9	37.5	3	1	4		96.83	215.1	7.74	0.147
10	37.5	3	1	8		91.88	175.5	7.35	0.006
11	37.5	3	2	4		95.97	208.3	7.68	0.005
12	37.5	3	2	8		91.68	164.7	7.33	0.012
13	37.5	9	1	4		95.55	208.6	22.93	0.007
14	37.5	9	1	8		91.05	177.0	21.85	0.005
15	37.5	9	2	4		95.55	201.1	22.93	0.022
16	37.5	9	2	8		91.25	166.5	21.90	0.009
17	12.5	6	1.5	6	Star design	89.61	160.1	43.01	0.005
18	37.5	6	1.5	6		91.08	167.3	14.57	0.122
19	25	3	1.5	6		92.87	182.5	11.14	0.130
20	25	9	1.5	6		91.02	181	32.77	0.071
21	25	6	1	6		90.35	187.9	21.68	0.094
22	25	6	2	6		91.35	154.5	21.92	0.011
23	25	6	1.5	4		95.87	207.2	23.01	0.008
24	25	6	1.5	8		91.8	177.1	22.03	0.036
25	25	6	1.5	6	Center points (n=3)	92.35	190.7	22.16	0.005
26	25	6	1.5	6		92.42	191.2	22.18	0.006
27	25	6	1.5	6		92.38	190.3	22.17	0.005

PLGA poly(D,L-lactic-co-glycolic acid), *PVA* polyvinyl alcohol, *EE* encapsulation efficiency, *DL* drug loading, *PDI* polydispersity index

Table III. ANOVA Results in Screening Design Plackett–Burman for EE, Particle Size, and DL

	Experimental response										
	EE (%)				Particle size (nm)				DL (%; *w/w*)			
Source	*df*	Sum of squares	*F* value	*P* value	*df*	Sum of squares	*F* value	*P* value	*df*	Sum of squares	*F* value	*P* value
Model	6	2,312.17	3,082.89	0.0138*	6	455.79	6,752.41	0.0093*	6	1,141.25	58.05	0.1001
1. PLGA (mg/ml)	1	2,125.52	17,004.20	0.0049*	1	3.25	289.00	0.0374*	1	317.32	96.84	0.0645
2. Drug (mg/ml)	1	22.56	180.50	0.0473*	1	0.30	26.89	0.1213	1	425.18	129.76	0.0557
3. Water volume (ml)	1	8.00	64.00	0.0792	1	21.45	1,906.78	0.0146*	1	3.26	0.99	0.5010
4. PVA (%)	1	7.84	62.72	0.0800	1	207.36	18,432.00	0.0047*	1	24.05	7.34	0.2251
5. Sonication power (W)	1	3.38	27.04	0.1210	1	3.00	266.78	0.0389*	1	5.46	1.67	0.4197
6. Sonication time (min)	1	20.70	165.62	0.0494*	1	26.01	2,312.00	0.0132*	1	0.30	0.09	0.8129
Error	1	0.13			1	0.01			1	3.28		
Corrected total	7	2,312.30			7	455.80			7	1,144.52		

PLGA poly(D,L-lactic-co-glycolic acid), *PVA* polyvinyl alcohol, *EE* encapsulation efficiency, *DL* drug loading, *PDI* polydispersity index
*α<0.05, significance level

(runs, 17–24) with a distance $\pm\alpha$ (α=1 for face CCD) away from the center, were added for constructing more efficient prediction models of prediction. Three center points (runs, 25–27) were added for testing experimental reproducibility (25).

Statistical Analysis

In this study, all data are expressed as means±standard deviation (SD; n=3). Statistical analyses were performed using SAS software 9.3 version (Cary, NC, USA). The response surface regression procedure was selected to find the coefficients and to plot contour and response surface. Analysis of contour and surface plots obtained was used for optimization and determination of interaction coefficients among various factors (26). A mathematical expression of experimental designs is represented in a polynomial equation (Eq. 3):

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \cdots + \alpha_{12} X_{12} + \alpha_{13} X_{13} + \alpha_{23} X_{23} + \cdots \quad (3)$$

Table IV. The Regression Coefficients in Screening Design Plackett–Burman for EE, Particle Size, and DL

	Experimental response		
Coefficient	EE (%)	Particle size (nm)	DL (%, *w/w*)
k_0	48.900	233.980	10.383
k_1/PLGA (mg/ml)	1.304*	0.051*	−0.504
k_2/drug (mg/ml)	−0.792*	0.092	3.437
k_3/water volume (ml)	0.250	−0.409*	0.160
k_4/PVA (%)	2.800	−14.400*	4.904
k_5/sonication power (W)	−0.087	−0.082*	−0.110
k_6/sonication time (min)	−1.138*	−1.275*	−0.137

PLGA poly(D,L-lactic-co-glycolic acid), *PVA* polyvinyl alcohol, *EE* encapsulation efficiency, *DL* drug loading, *PDI* polydispersity index
*α<0.05, significance level

Y is the measured response of EE, particle size, and DL; α_0 indicates a constant representing the mean of dependent variable obtained in each experiment; α_i and α_{ij} are the linear effects of variables X_i and the first-order interactions between X_i and X_j, respectively; X_i (for i=1, 2, 3, …) represents independent coded level of variables; X_{ij} (for i,j =1, 2, 3, …, $i<j$) is the interaction coefficient between X_i and X_j; α_{ii} represents the quadratic effect of variables X_{ii}. In this polynomial equation, a positive parameter indicates a synergistic effect, where the output ascends with an increase in input variables. Conversely, a negative sign denotes an antagonistic effect where output descends with elevation in input variables (27). Two-way analysis of variance (ANOVA) can be applied to determine statistical significance of each model coefficient, and least significant difference as post hoc test was performed. Contour plots and surface response plots are diagrammatic representation of the values of the response. These plots are useful to project the magnitude of effects for each variable and interactions. It can also explain the relationship between independent variables and dependent responses. The reduced models were used to plot two-dimensional contour plots and three-dimensional response surface plots using a SAS software. Response surface methodology provides a mathematical trend that can find optimum level of experimental factors required for a given response (28). An additional formulation as the experimental checkpoint was also performed to compare with predicted optimum values.

Table V. Identified Critical Factors and Levels Investigated in Central Composite Design

	Transformed levels		
Critical factors	−1	0	1
X_A/PLGA (mg/ml)	12.5	25	37.5
X_B/drug (mg/ml)	3	6	9
X_C/PVA (%)	1	1.5	2
X_D/sonication time (min)	4	6	8

PLGA poly(D,L-lactic-co-glycolic acid), *PVA* polyvinyl alcohol

Table VI. ANOVA Results in Central Composite Design for Entrapment Efficiency (EE), Particle Size, and Drug Loading (DL)

	Experimental response											
	EE (%)				Particle size (nm)				DL (%; *w/w*)			
Source	*df*	Sum of squares	*F* value	*P* value	*df*	Sum of squares	*F* value	*P* value	*df*	Sum of squares	*F* value	*P* value
Model	14	316.48	25.79	<0.0001*	14	12,784.75	11.88	<0.0001	14	8,309.95	226.90	<0.0001*
X_A	1	33.18	37.86	<0.0001*	1	788.05	10.25	0.0076*	1	3,638.90	1,391.01	<0.0001*
X_B	1	7.68	8.77	0.0119*	1	72.00	0.94	0.3523	1	3,534.16	1,350.97	<0.0001*
X_C	1	0.17	0.19	0.6673	1	595.13	7.74	0.0166*	1	0.08	0.03	0.8675
X_D	1	224.72	256.39	<0.0001*	1	9,809.34	127.56	<0.0001*	1	32.83	12.55	0.0041*
X_{AB}	1	0.99	1.14	0.3076	1	11.73	0.153	0.7030	1	777.99	297.40	<0.0001*
X_{AC}	1	0.07	0.08	0.7880	1	0.08	0	0.9755	1	0.11	0.044	0.8382
X_{AD}	1	34.37	39.21	<0.0001*	1	520.98	6.77	0.0231*	1	19.43	7.43	0.0184*
X_{BC}	1	0.03	0.03	0.8651	1	2.18	0.03	0.8692	1	0.10	0.04	0.8500
X_{BD}	1	0.23	0.26	0.6193	1	59.68	0.78	0.3957	1	9.69	3.70	0.0783
X_{CD}	1	0.13	0.15	0.7092	1	0	0	0.9978	1	0.15	0.06	0.8124
X_A^2	1	4.41	5.03	0.0445*	1	461.85	6.01	0.0306*	1	119.10	45.53	<0.0001*
X_B^2	1	0.22	0.25	0.6279	1	55.56	0.78	0.4120	1	0	0	0.9773
X_C^2	1	1.66	1.90	0.1933	1	89.57	1.16	0.3017	1	0.09	0.03	0.8581
X_D^2	1	12.22	13.95	0.0028*	1	582.29	7.57	0.0175*	1	0.74	0.28	0.6050
Error	12	10.52			12	922.79			12	31.39		
Corrected total	26	327.00			26	13,707.54			26	8,341.34		

Regression analysis
*α<0.05, significance level

RESULTS AND DISCUSSION

Screening Experimental Design

A structured experimental design matrix with only eight experiments was built according to Plackett–Burman design to prescreen the influence of six parameters at their lowest and highest factor levels. The input factor conditions and resulting experimental response data for eight prescreening experimental runs are shown in Table I. The experimental responses considered included EE, particle size, and DL. The model to determine the response coefficients can be expressed as Eq. 4:

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_4 X_4 + \alpha_5 X_5 + \alpha_6 X_6 \quad (4)$$

X_1 is the concentration of PLGA (mg/ml); X_2 is the concentration of drug (mg/ml); X_3 is volume of water; X_4 represents concentration of PVA (percent); X_5 is sonication

Table VII. The Regression Coefficients in Central Composite Design, *P* values, R^2, and Adjusted R^2 for Full Model of Entrapment Efficiency (EE), Particle Size, and Drug Loading (DL)

	Experimental response		
Coefficient	EE (%)	Particle size (nm)	DL (%; *w/w*)
α_0	91.898	181.646	22.046
α_A	1.358*	6.617*	−14.218*
α_B	−0.653*	−2.000	14.012*
α_C	−0.097	−5.750*	−0.065
α_D	−3.533*	−23.344*	−1.351*
α_{AB}	0.249	0.856	−6.973*
α_{AC}	0.064	−0.069	0.084
α_{AD}	1.466*	5.706*	1.102*
α_{BC}	−0.041	−0.368	−0.078
α_{BD}	−0.119	1.931	−0.778
α_{CD}	−0.089	0.006	−0.098
α_A^2	−1.310*	−13.402*	6.806*
α_B^2	0.290	4.648	−0.029
α_C^2	−0.805	−5.902	−0.184
α_D^2	2.180*	15.048*	0.536
R^2	0.9678	0.9327	0.9962
Adjusted R^2	0.9303	0.8541	0.9918
CV	1.016	4.821	6.036

*α<0.05, significance level
CV coefficient of variation

Table VIII. The Regression Coefficients in Central Composite Design, *P* values, R^2, and Adjusted R^2 for Reduced Model of Entrapment Efficiency (EE), Particle Size, and Drug Loading (DL) of NP

	Experimental response		
Coefficient	EE (%)	Particle size (nm)	DL (%; *w/w*)
α_0	91.829	181.479	22.118
α_A	1.358	6.617	−14.218
α_B	−0.653		14.012
α_C		−5.750	
α_D	−3.533	−23.344	−1.351
α_{AB}			−6.973
α_{AD}	1.466	5.706	1.102
α_A^2	−1.515	−13.903	7.021
α_D^2	1.975	14.547	
F value of model	75.61	35.30	653.93
P value of model	<0.0001*	<0.0001*	<0.0001*
R^2	0.9578	0.9137	0.9949
Adjusted R^2	0.9451	0.8878	0.9934
CV	0.902	4.227	5.427

*α<0.05, significance level
NP nanoparticles, *CV* coefficient of variation

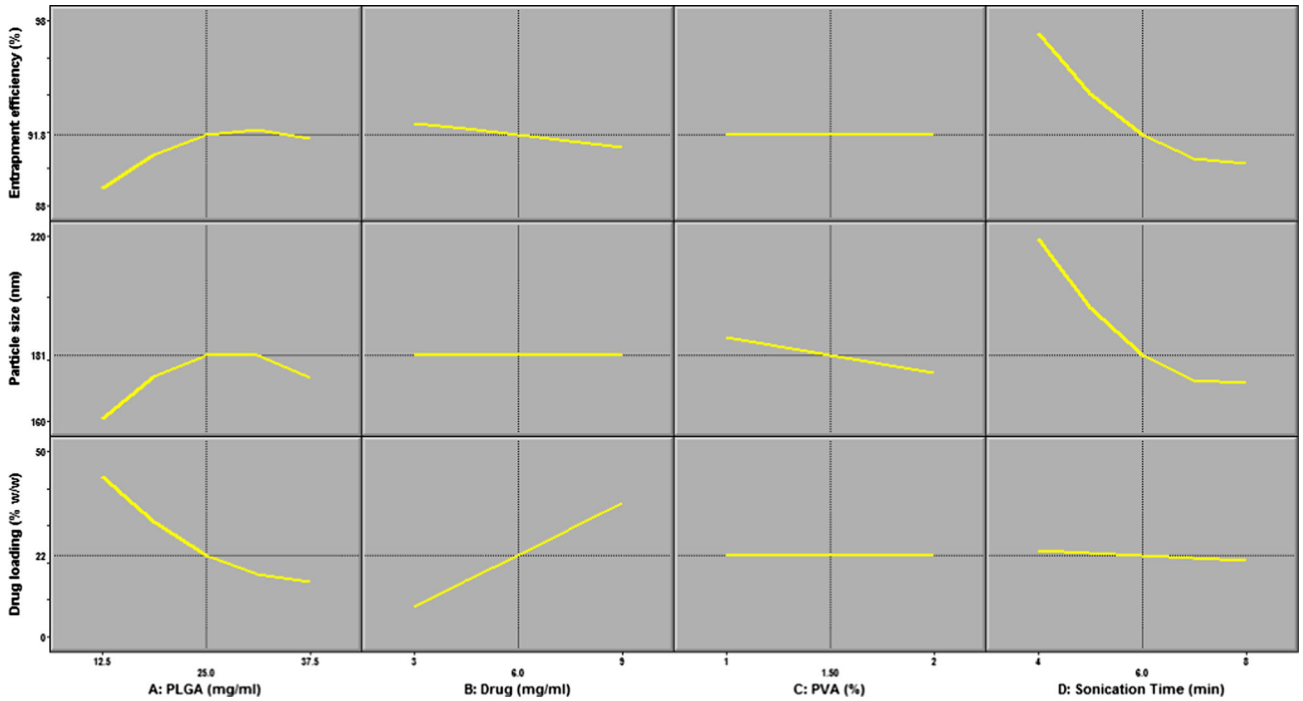

Fig. 1. Main effects plot for EE, particle size, and DL of NP

power; and X_6 denotes sonication time (minutes) in coded values. Each experimental response Y can be represented by an individual quadratic equation.

Analysis of data was carried out by ANOVA, and the individual parameter was evaluated with F test as shown in Table III. The corresponding regression coefficients were summarized in Table IV. The results from Table III indicate that concentration of PLGA and drug and the sonication time exert significant effects on EE. As expected, an addition of more PLGA enhanced EE, whereas an increase in drug and sonication time led to lower EE (Table IV). In addition, the particle size diminished with rise in several factors including PVA, intensity, and duration of sonication. However, PLGA produced a significant synergistic effect on particle size (Table IV). None of the factors appeared to exert significant influence on DL by prescreening experimental design shown in Table III. Therefore, four critical factors including PLGA, drug and PVA levels, and sonication time were first selected by Plackett–Burman design. It was further optimized by another experimental design for obtaining desirable responses (Table V).

The screening design Plackett–Burman only evaluates the effects of individual variables but not the interactions between variables and their impact on each response (29). However, this step is necessary to simplify various variables. It also enables us to further focus on more detailed examination and optimization of several critical factors.

Central Composite Design

CCD can provide minimal experimental runs to investigate all the effects and interactions between the four experimental factors from prescreening study (30). In this study, all the models of CCD permit to find optimal operating conditions with high EE, small particle size, and great DL, which can be controlled by adjusting different variables. The matrix of the CCD and resulting experimental responses are provided in Table II. The results of the experimental design are analyzed with SAS software, which can provide considerable useful information and confirm the utility of statistical design in conducting experiments. The ANOVA results and regression coefficients for each response based on 27 runs in this design are summarized in Tables VI and VII, respectively. The following quadratic model is carried out to fit the data:

$$Y = \alpha_0 + \alpha_A X_A + \alpha_B X_B + \alpha_c X_c + \alpha_d X_d + \alpha_{AB} X_{AB} + \alpha_{AC} X_{AC} + \alpha_{AD} X_{AD} + \alpha_{BC} X_{BC} + \alpha_{BD} X_{BD} + \alpha_{CD} X_{CD} + \alpha_{A^2} X_A^2 + \alpha_{B^2} X_B^2 + \alpha_{C^2} X_C^2 + \alpha_{D^2} X_D^2 \quad (5)$$

X_A is the concentration of PLGA (milligrams per milliliter); X_B denotes the concentration of drug (milligrams per milliliter); X_C represents concentration of PVA (percent); and X_D is sonication time (minutes) in coded values. Each experimental response Y can be represented by an individual quadratic equation.

Effect of Formulation Variable on EE

The full model for Y_1 (EE %) is given by Eq. 6.

$$Y_1(\text{EE }\%) = 91.898 + 1.358X_A - 0.653X_B - 0.097X_C - 3.533X_D + 0.249X_{AB} + 0.064X_{AC} + 1.466X_{AD} - 0.041X_{BC} - 0.119X_{BD} - 0.089X_{CD} - 1.310X_A^2 + 0.290X_B^2 - 0.805X_C^2 + 2.180X_D^2 \quad (6)$$

with $R^2 = 0.9678$, adjusted $R^2 = 0.9303$, and CV = 1.016

The model coefficients estimated by quadratic model for EE are given in Table VII. The regression coefficients with

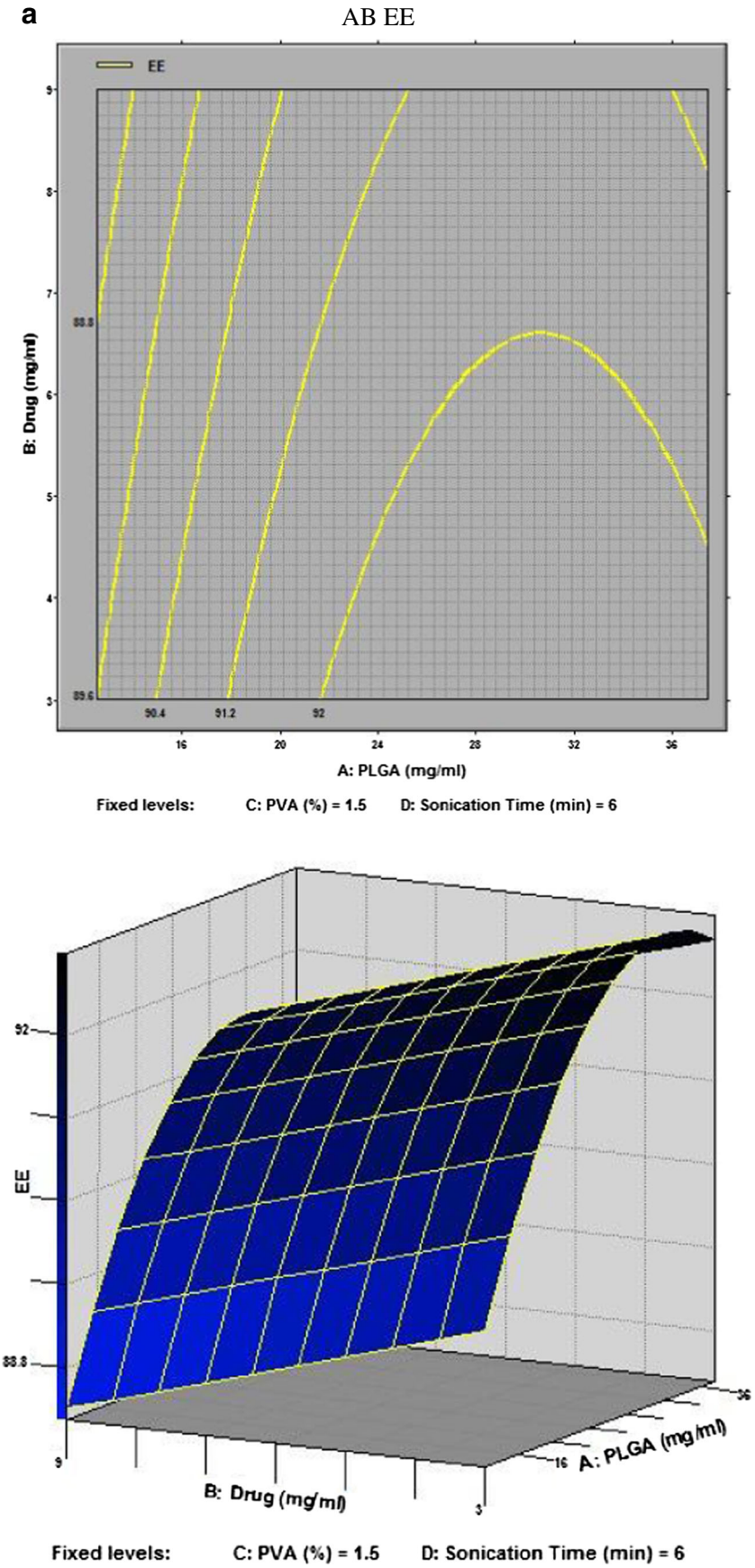

Fig. 2. Contour plots and response surface plots of EE of NP. **a** X_A and X_B, X_C=1.5, and X_D=6; **b** X_A and X_D, X_B=6, and X_C=1.5; and **c** X_B and X_D, X_A=25, and X_C=1.5

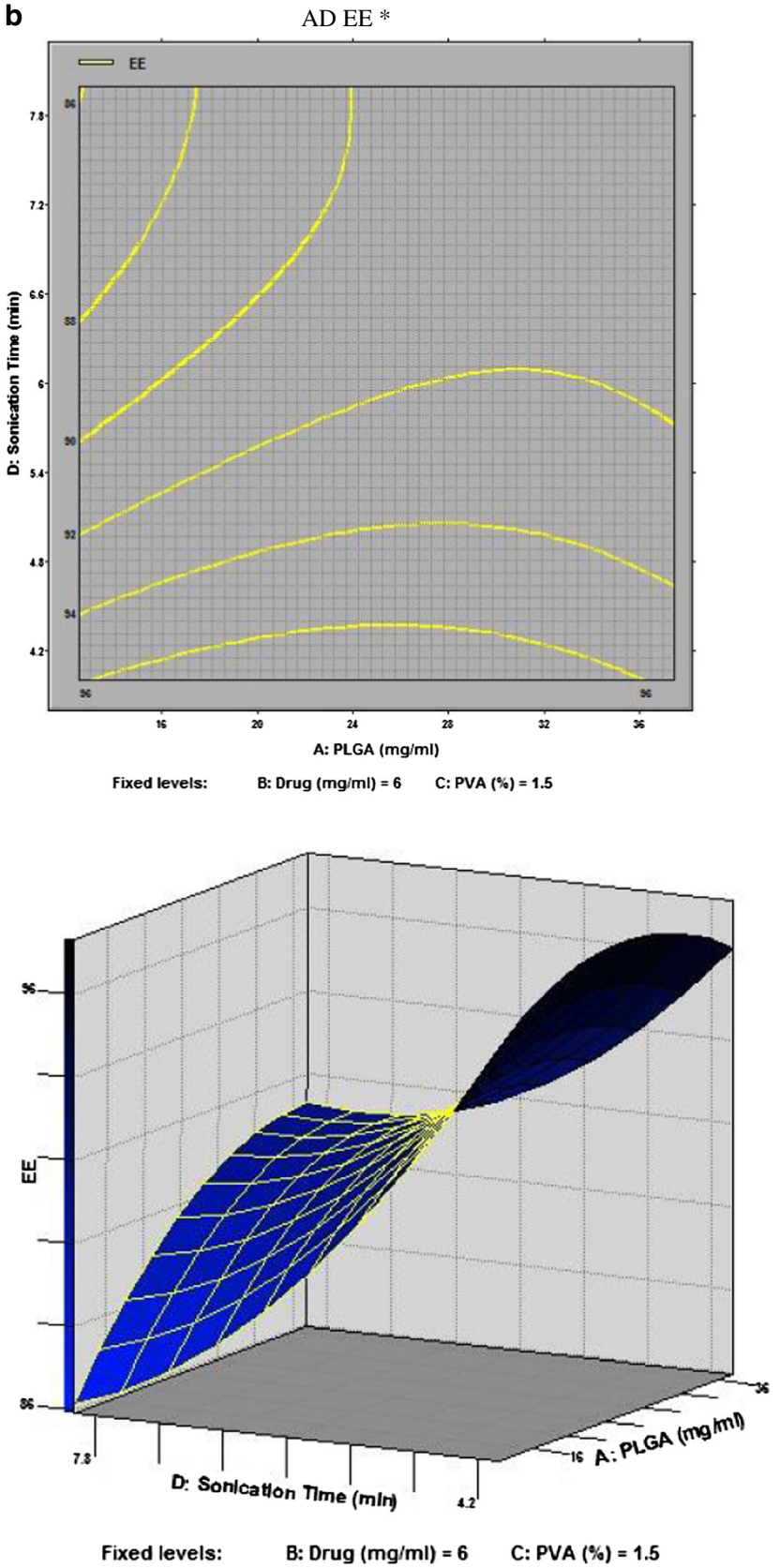

Fig. 2. (continued)

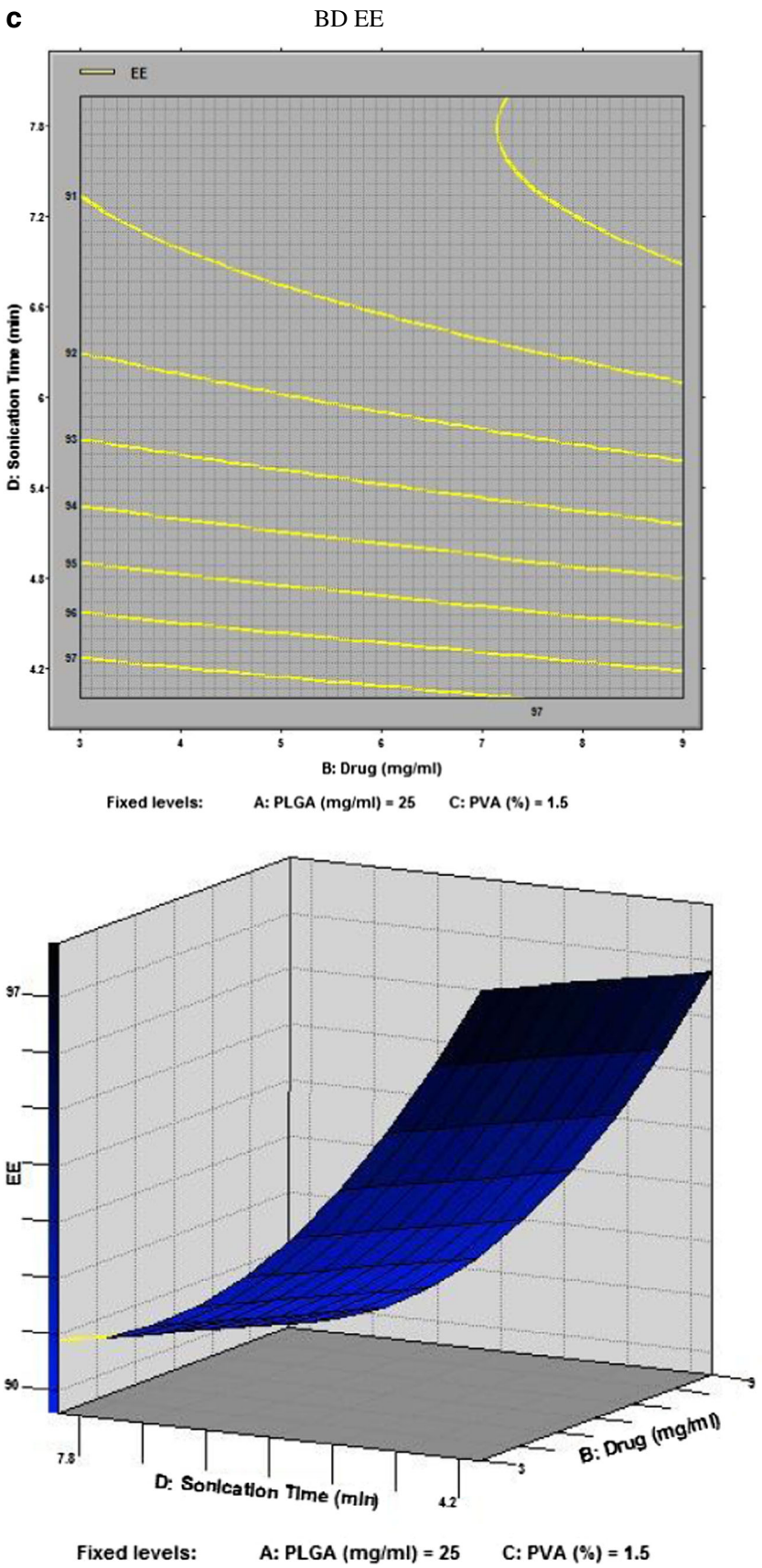

Fig. 2. (continued)

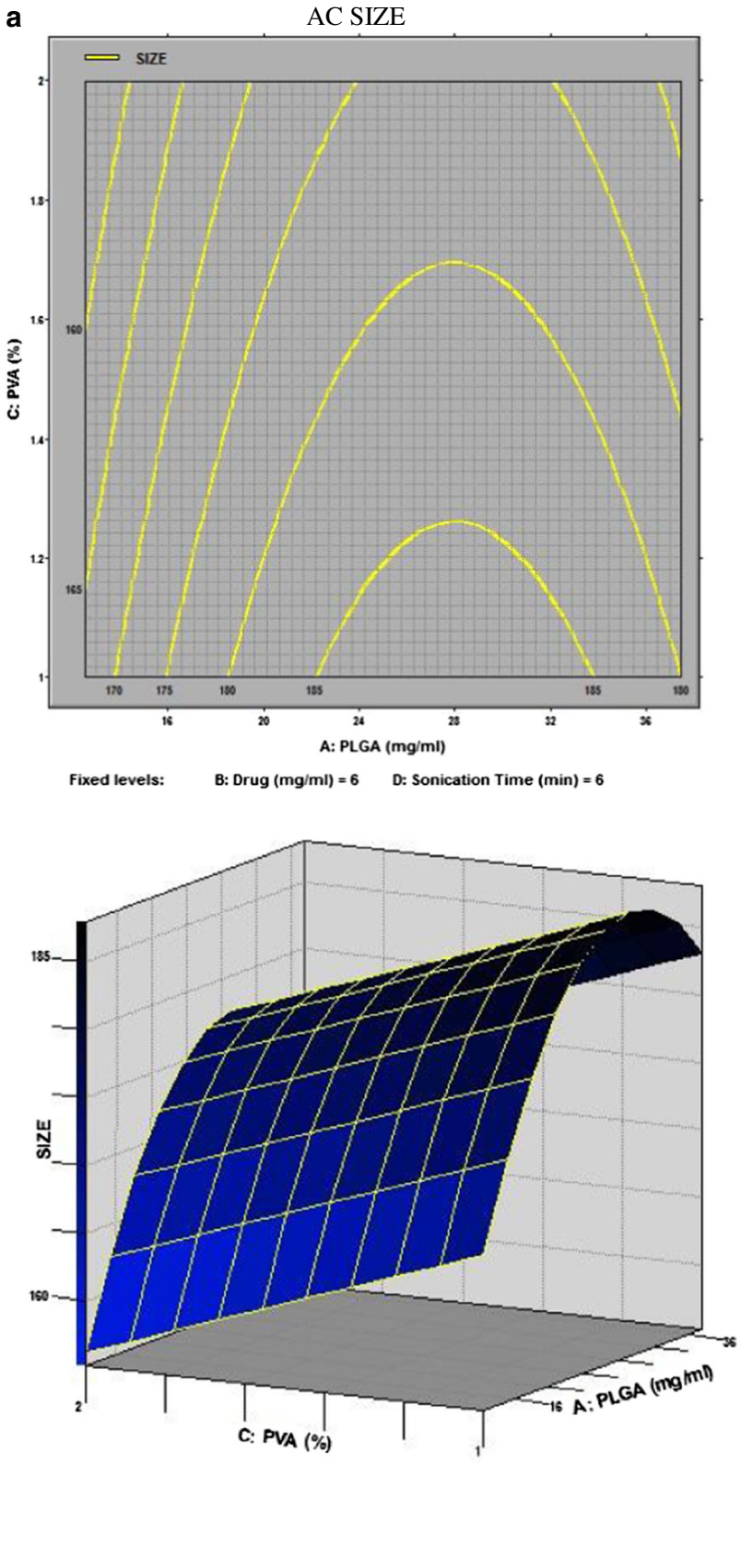

Fig. 3. Contour plots and response surface plots of particle size of NP. **a** X_A and X_C, X_B=6, and X_D=6; **b** X_A and X_D, X_B=6, and X_C=1.5; and **c** X_C and X_D, X_A=25, and X_B=6

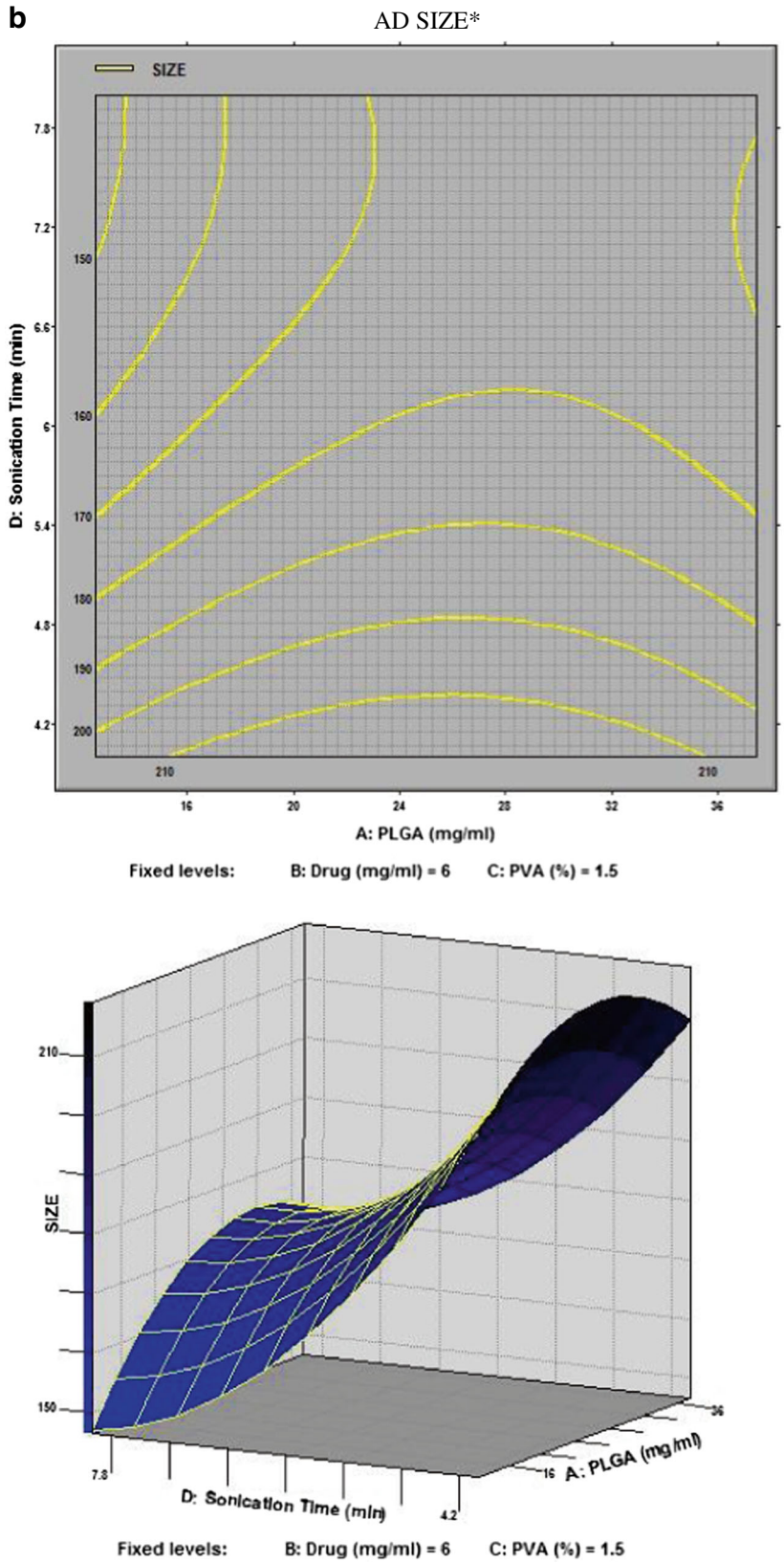

Fig. 3. (continued)

P value<0.05 are highly significant, whereas the terms having nonsignificant response coefficients with a *P* value>0.05 are least contributing in the prediction of EE and hence are removed from the full model to generate the reduced model equation.

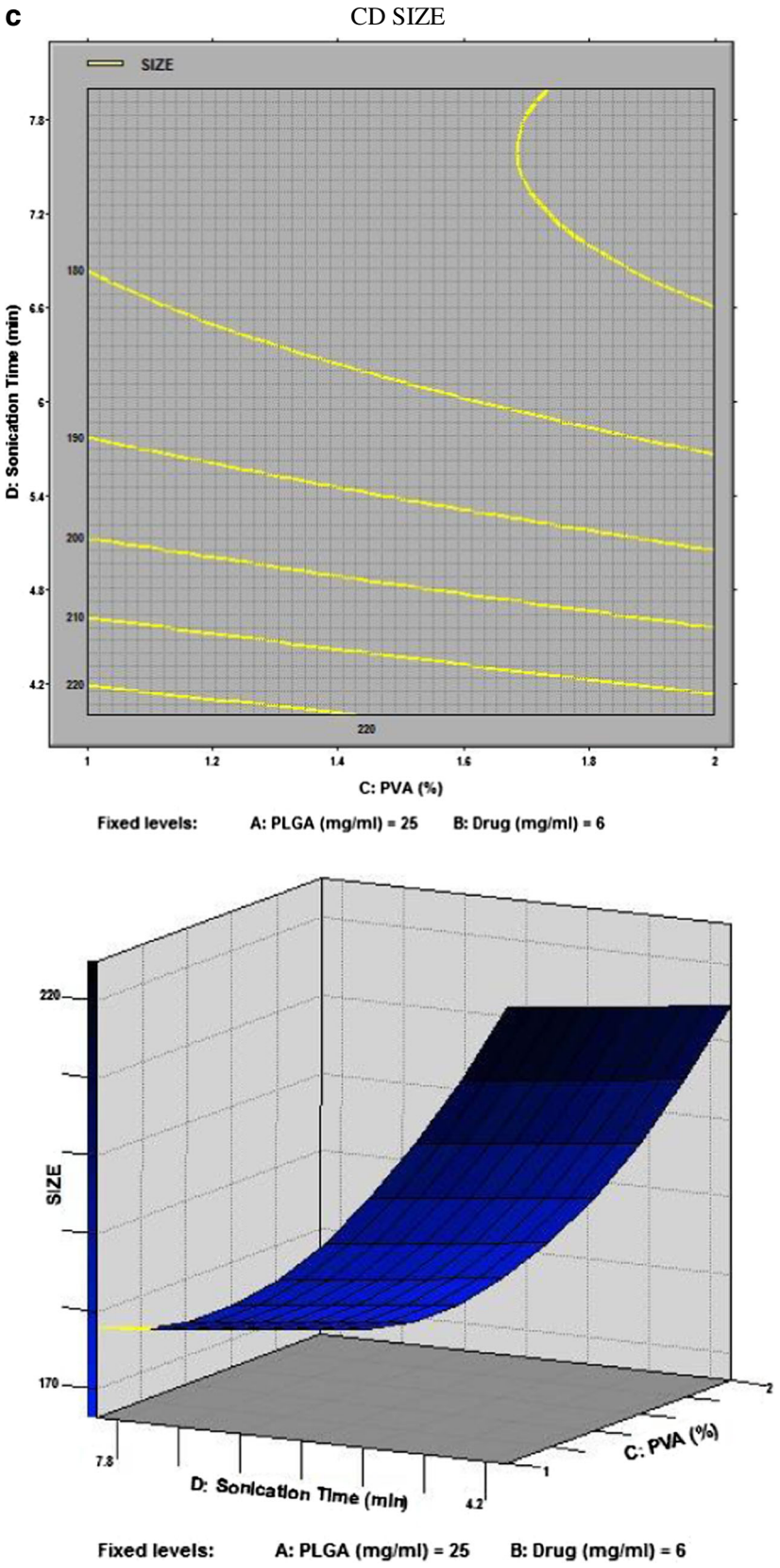

Fig. 3. (continued)

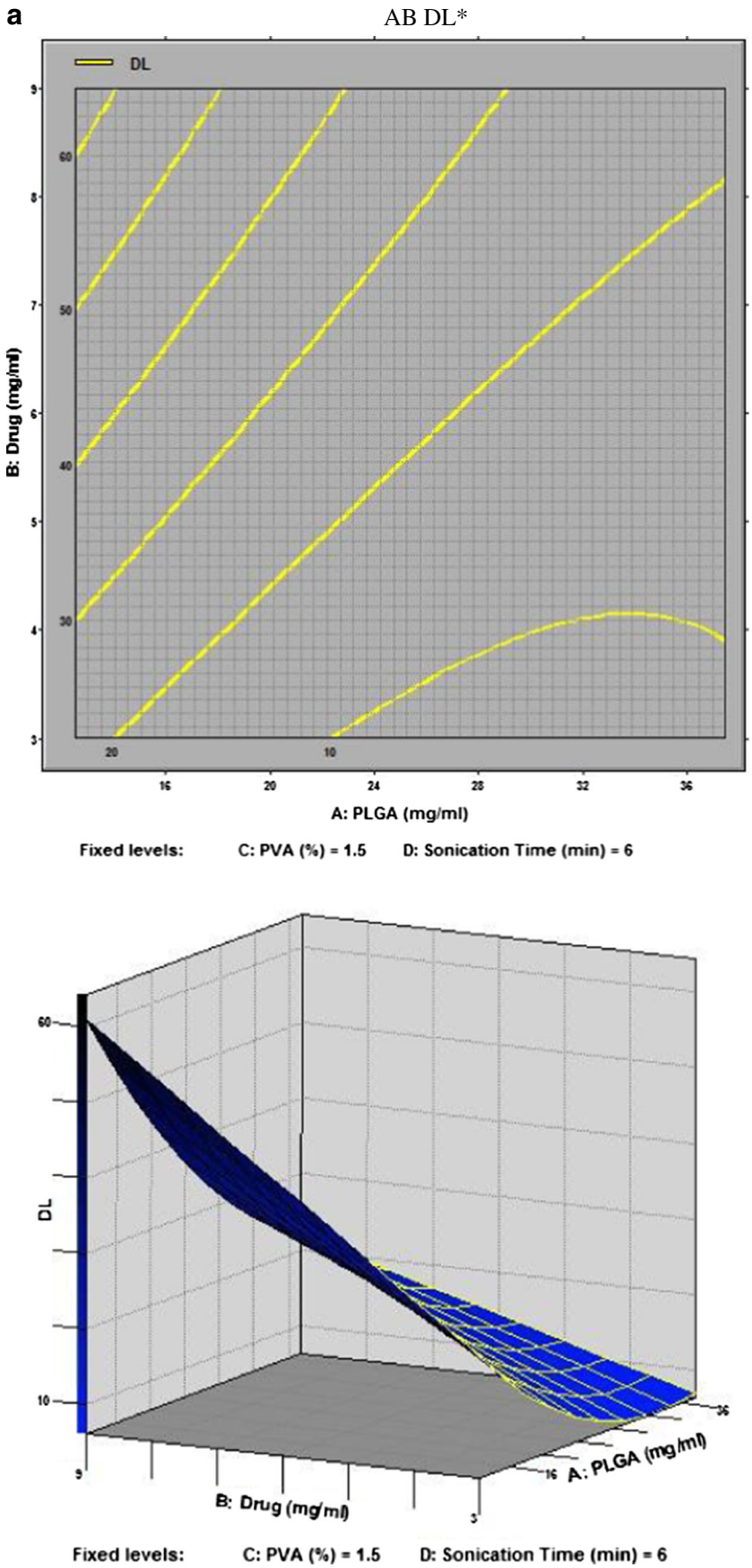

Fig. 4. Contour plots and response surface plots of DL of NP. **a** X_A and X_B, X_C=1.5, and X_D=6; **b** X_A and X_D, X_B=6, and X_C=1.5; and **c** X_B and X_D, X_A=25, and X_C=1.5

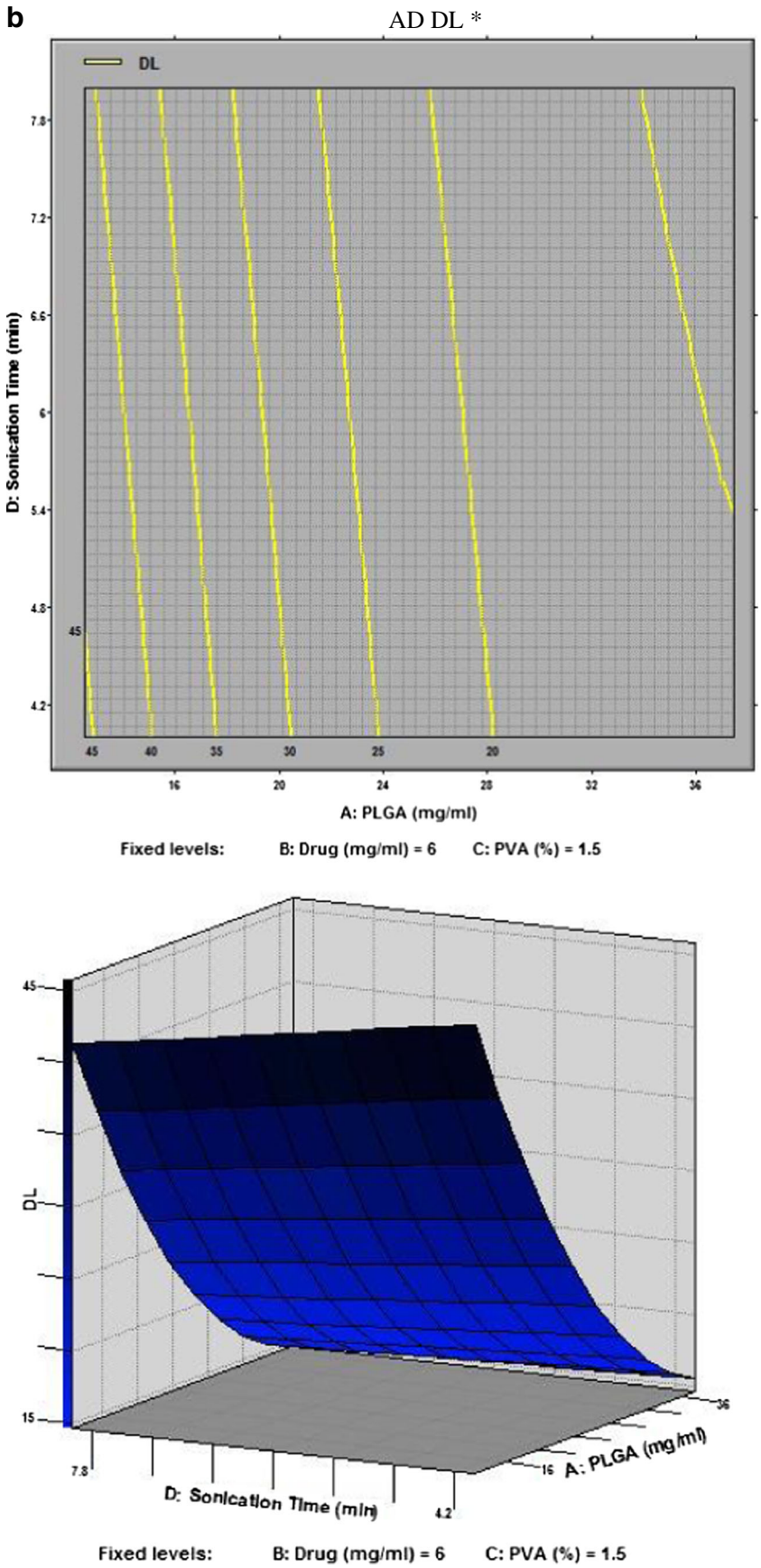

Fig. 4. (continued)

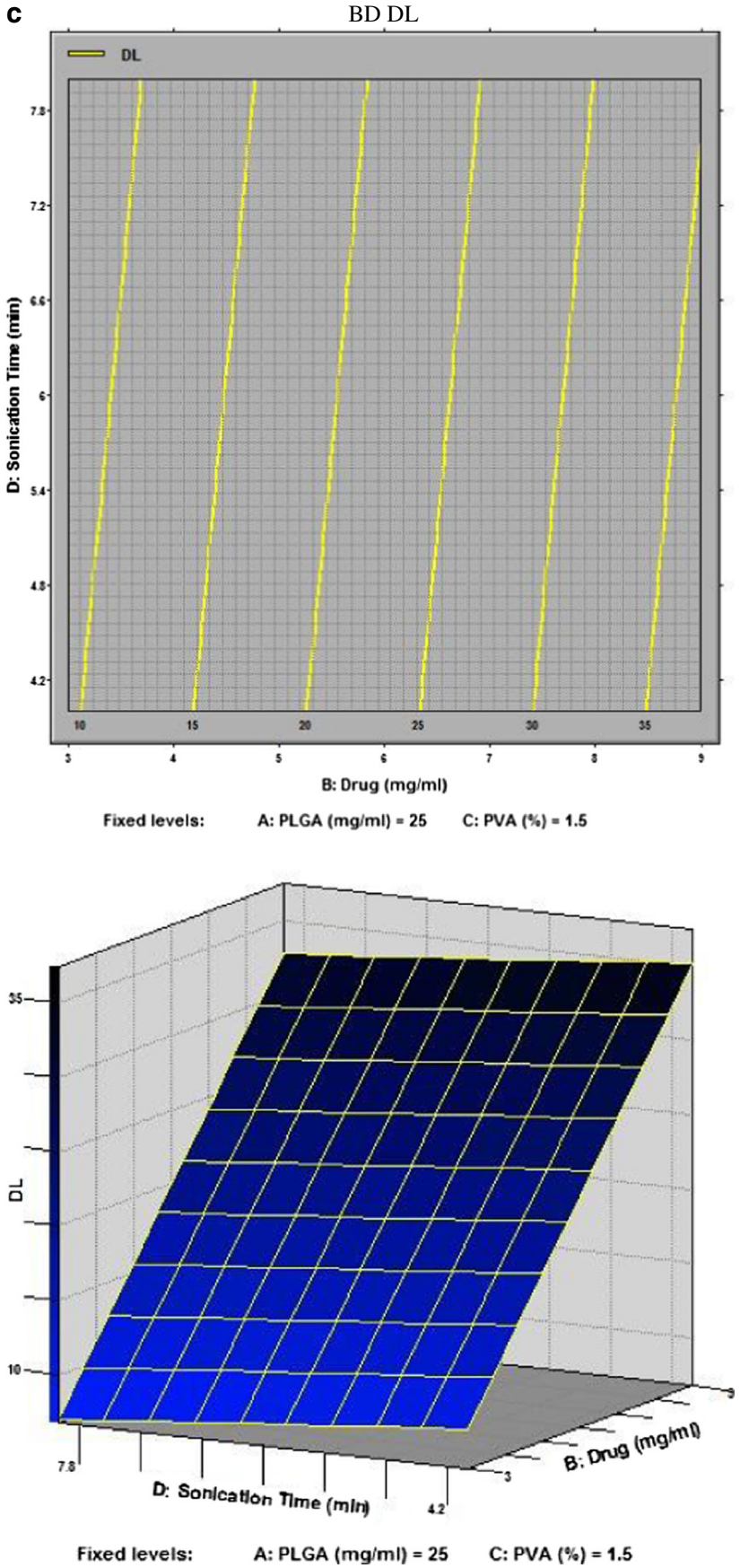

Fig. 4. (continued)

The following polynomial equation indicates the reduced model having significant coefficients for Y_1 (EE %).

$$Y_1(\text{EE }\%) = 91.829 + 1.358X_A - 0.653X_B - 3.533X_D + 1.466X_{AD} - 1.515X_A^2 + 1.975X_D^2 \quad (7)$$

with R^2=0.9578, adjusted R^2=0.9451, and CV=0.902

In Eq. 7, the quadratic model is found to be significant with an F value of 25.79 (P value<0.0001), which indicates that response Y_1 and the set of X variables are significantly related (Table VI). F value of the reduced model is 75.61 with a very low probability value (P value <0.0001), demonstrating that the reduced model is also highly significant. The determination coefficient (R^2) of full model is 0.9678, higher than R^2 of 0.9578 in reduced model, because R^2 value is always higher by adding a variable to the model, regardless of whether the additional variable is significant or not (31). A high R^2 value indicates that 96.78% of variation in EE was explained by the regression of preparation variables. However, a large value of R^2 is not necessary to confirm the good fit for the regression model. In such cases, the adjusted R^2 value can be selected to evaluate the good fit of the model. Removal of nonsignificant terms increases the adjusted R^2 value (32). The adjusted R^2 value (0.9451) in reduced model for EE is higher than that (0.9303) in the full model. This result indicates that reduced model fits the observed data quite well. Compared with the full model, the results for reduced model showed that R^2 value is as high as the adjusted R^2 value, which indicates a higher significance of the reduced model (Table VIII) (33). In addition, the value of the CV (0.902) is relatively low, indicating improved precision and reliability of the experiments (34).

It is obvious that the concentration of PLGA, drug, and sonication time are the main factors with significant effects on the EE of NP as shown in Fig. 1. The plots also indicated the synergistic effect of PLGA and antagonistic effects of drug and sonication time. The contour plots and response surface curves for EE as a function of preparation factors are constructed by fixing two of the variables (Fig. 2). This gives a diagrammatic representation of relationship between the experimental responses and input variables. As shown in Fig. 2, EE changes sharply by varying PLGA and sonication time. As we expected, a polymer concentration increase led to a concentration-dependent increase in EE, whereas the sonication time decreased the EE. However, a higher level of interactions between PLGA and sonication time shows a higher EE of NP. A significant (P value<0.0001) synergistic interaction between PLGA and sonication time are found (Tables VI and VII), which matched the pattern of interaction depicted in plots and there is no significant interaction between other variables for EE. The quadratic regression coefficients α_{A^2} and α_{D^2} are statistically significant. The quadratic effect of sonication time shows significant (P value=0.0028) synergistic influence on EE of NP. A significant (P value=0.0445) antagonistic influence of quadratic effect of PLGA on EE is evident (Tables VI and VII).

Effect of Formulation Variable on Particle Size

The full model for Y_2 (particle size) is given by Eq. 8.

$$Y_2(\text{Particle size}) = 181.646 + 6.617X_A - 2.000X_B - 5.750X_C - 23.344X_D + 0.856X_{AB} - 0.069X_{AC} + 5.706X_{AD} - 0.368X_{BC} + 1.931X_{BD} + 0.006X_{CD} - 13.402X_A^2 + 4.648X_B^2 - 5.902X_C^2 + 15.048X_D^2 \quad (8)$$

with R^2=0.9327, adjusted R^2=0.8541, and CV=4.821

The model coefficients estimated by quadratic model is depicted in Table VII. The following polynomial in Eq. 9 indicates the reduced model by removal of nonsignificant response coefficients with P value>0.05 for Y_2 (particle size).

$$Y_2(\text{Particle size}) = 181.479 + 6.617X_A - 5.750X_C - 23.344X_D + 5.706X_{AD} - 13.903X_A^2 + +14.547X_D^2 \quad (9)$$

with R^2=0.9137, adjusted R^2=0.8878, and CV=4.227

For the particle size, the model F value of 11.88 with a very low probability value (P value<0.0001) implies a very high significance for the full regression model (Table VI). Model F value of the reduced model is 35.30 with a very low probability value (P value<0.0001), demonstrating that the reduced model is also highly significant. R^2 values of full and reduced models are 0.9327 and 0.9137, respectively, indicating excellent correlation between the independent variables in both models. The adjusted R^2 values of full and reduced models were 0.8541 and 0.8878, respectively, indicating that the reduced model with higher significance is a better model for fitting (Table VIII). At the same time, a relatively low value of CV (4.227) in the reduced model has greater precision and reliability of the conducted experiments, compared with CV (4.821) in full model.

The results in Table VI show that particle size is highly dependent on the concentration of PLGA, PVA, and sonication time. Figure 1 demonstrates an increasing effect of PLGA but a lowering effect of PVA and sonication time. The possible interactions between these factors for particle size were also investigated. This is reflected by pattern of the contour lines and response surface curves in Fig. 3. A significant (P value= 0.0231) synergistic interaction between PLGA and sonication time were also found for particle size however no such interactions between other variables are significant for particle size. The quadratic regression coefficients α_{A^2} and α_{D^2} are statistically significant. The quadratic effect of sonication time also suggest significant (P value=0.0175) synergistic influence on the particle size of NP. A significant (P

Table IX. Comparison Between the Model Constant and the Average Experimental Results in the Center of the Domain (n=3)

Coefficient	Experimental response		
	EE (%)	Particle size (nm)	DL (%; *w/w*)
α_0	91.898	181.646	22.046
Average result	92.38	190.73	22.17

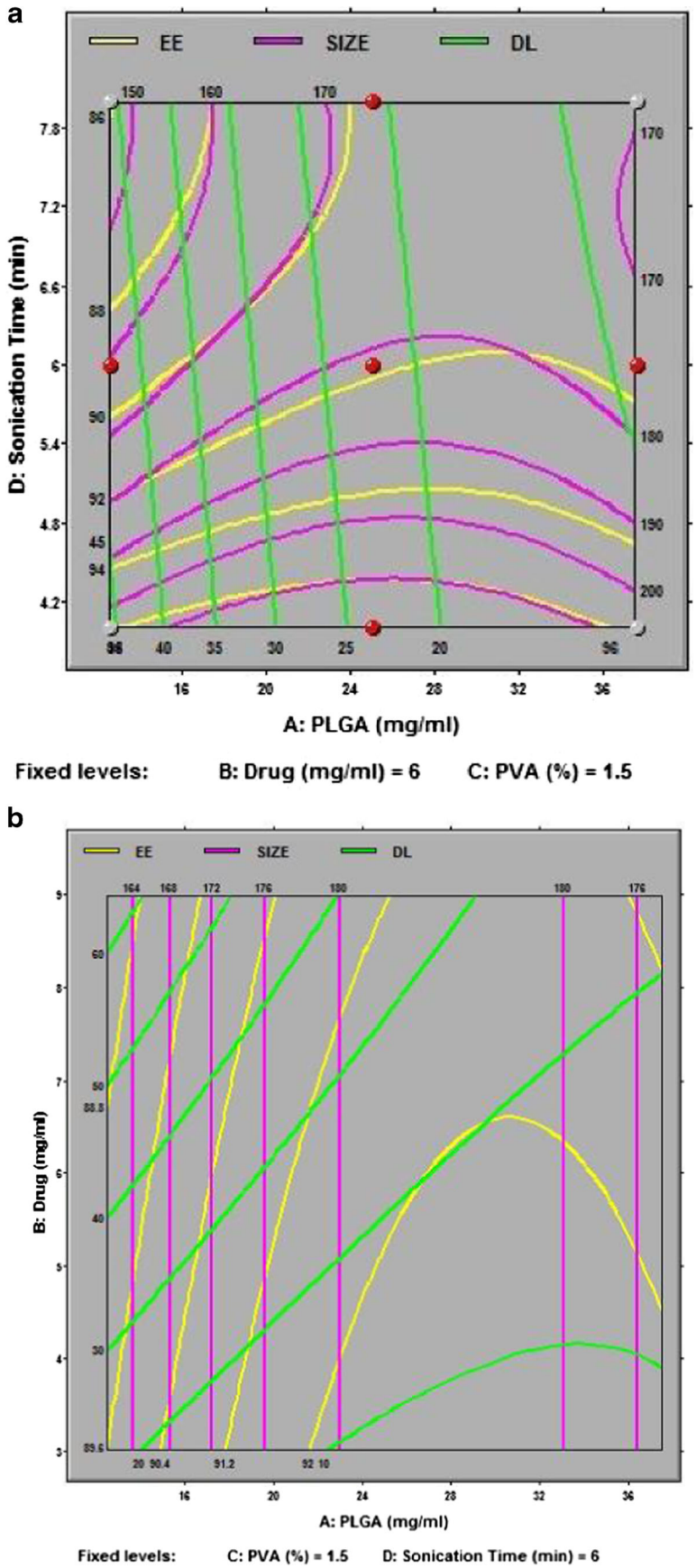

Fig. 5. Overlaid contour plots of NP with independent variables. **a** X_A and X_D, X_B=6, and X_C=1.5 and **b** X_C=1.5 and X_D=6

value=0.0306) antagonistic quadratic effect of PLGA on the particle size was observed.

Effect of Formulation Variable on DL

The full model for Y_3 (DL) is given by Eq. 10.

$$Y_3(\mathrm{DL}) = 22.046 - 14.218X_A + 14.012X_B - 0.065X_C - 1.351X_D - 6.973X_{AB} + 0.084X_{AC} + 1.102X_{AD} - 0.078X_{BC} - 0.778X_{BD} - 0.098X_{CD} + 6.806X_A^2 - 0.029X_B^2 - 0.184X_C^2 + 0.536X_D^2 \quad (10)$$

with R^2=0.9962, adjusted R^2=0.9918, and CV=6.036

The model coefficients estimated by quadratic model for DL are shown in Table VII. The following polynomial Eq. 11 indicates the reduced model for Y_3 (DL).

$$Y_3(\mathrm{DL}) = 22.118 - 14.218X_A + 14.012X_B - 1.351X_D - 6.973X_{AB} + 1.102X_{AD} + 7.021X_A^2 \quad (11)$$

with R^2=0.9949, adjusted R^2=0.9934, and CV=5.427

In full and reduced regression models for DL, model F values are 226.90 and 653.93, respectively, implying that both models are highly significant with P values less than 0.0001 as shown in Table VI. In both models, R^2 values (0.9962 and 0.9949, respectively) and adjusted R^2 values (0.9918 and 0.9934, respectively) are high (>0.99), indicating an excellent correlation between independent variables and good fit for the regression model (Tables VII and VIII). Since, adjusted R^2 values are improved with a relatively low value of CV (5.427). It was obtained by omitting the terms with P value>0.05. Therefore, a reduced model is chosen for DL.

In the reduced model of DL, the PLGA, drug, and sonication time exhibit significant effect (Table VI). The main effect plots in Fig. 1 illustrate the increasing effect of drug and decreasing effect of PLGA and sonication time. In the contour plot and surface response plots for DL, interaction between PLGA and drug and that between PLGA and sonication are depicted in Fig. 4. DL changed significantly by varying concentration of PLGA. However, such change came in a relatively gradual fashion as viewed from the drug axis or sonication time axis. This result indicates that the PLGA is the most important factor for interaction effect on DL. It is concluded that interaction between PLGA and sonication has a synergistic effect on DL where more interactions lead to higher DL. By contrast, higher interactions between PLGA and drug caused an antagonistic effect on DL, resulting in lower DL. No significant interactions between other variables were found. The quadratic regression coefficient α_{A^2} is statistically significant. The quadratic effect of PLGA also shows significant (P value<0.0001) synergistic influence on DL of NP.

Optimization of Formulation

The three center-point experiments resulted in minor differences between model constants and average results for EE and DL (Table IX). However, a significant difference between the model constants and the average results for particle size was observed, indicating that the calculated model does not always generate good prediction of responses everywhere in the experimental domain.

Table X. Comparison of the Observed and Predicted Values of the Response Variables of Optimized Formulation

Experimental response	Predicted values	Experimental values	Bias* (%)
EE (%)	89.38	90.60	−1.37
Particle size (nm)	163.9	164.3	−0.24
DL (%; *w/w*)	64.83	64.35	0.73

Bias was calculated as (predicted observed value)/predicted value× 100%

*α<0.05, significance level

The qualitative and quantitative influence of independent factors on responses of EE, particle size, and DL may clearly be interpreted from the aforementioned models. Consequently, they may be further used for selection of optimized formulation by SAS software, which offers to vary each variable simultaneously and allows to develop possible optimum selections with their respective desirability value. A lack of adequate fit of models will cause poor or misleading results to the continuing examination and optimization of the fitted response surface. The residual analysis is one method to check model adequacy. By constructing a normal probability plot of the residuals form the least square fit, the normality can be confirmed (35). In this study, the normality is satisfactory as all residual plots are distributed along a straight line. This means the confidences for the fitness of the regression equations to the observed values are more than 95% for all responses.

The canonical analysis in the SAS software is a mathematical tool for simplifying a second-order polynomial model and simultaneously observing the extreme values of several response surface models. Overlaid contour plots of NP with independent variables were constructed by fixing two of the variables. The plots demonstrated a trend of optimum level for 4 factors (Fig. 5). According to our criteria for higher EE, lower particle size, and higher DL, the optimized PLGA, drug, PVA, and sonication time under canonical analysis were selected at 12.5 mg/ml, 9 mg/ml, 1.8%, and 5.6 min, respectively. EE, particle size, and DL of optimized formulation were predicted to be 89.38%, 163.9 nm, and 64.83%, respectively (Table X). An extra design checkpoint formulation under optimized factors was performed to compare with the predicted values. As shown in Table X, the bias were around −1.37%, −0.24%, and 0.73% for each respective response, indicating the validity of generated models with no statistically significant difference and good correlation between predicted and experimental values.

CONCLUSIONS

In this study, experimental designs were involved to investigate the effects of formulation and process variables on the resulting EE, particle size, and DL. The Plackett–Burman as a prescreening design allowed to select critical factors from various variables. The application of CCD proved to be a useful tool for optimizing HB-loaded PLGA NP prepared by emulsion solvent evaporation technique. An analysis of these results was processed by polynomial equations and multiple regression. These results of statistical analysis demonstrate

that concentration of PLGA and drug and sonication time appear to be the crucial parameters for the EE and DL, whereas concentration of PLGA, sonication power, and time are important for the particle size. According to our studied factors, the selected optimum formulation with 90.6% of EE, 164.3 nm of particle size with PDI of 0.005, and 64.35% of DL was processed by 12.5 mg/ml of PLGA, 9 mg/ml of drug, 1.8% of PVA, and 5.6 min of sonication time. Observed response is in close agreement with the predicted values of the optimized formulation. Therefore, the statistical experimental design methodology has clearly shown the feasibility of the optimization procedure in developing HB-loaded NP.

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