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Research Article

Optimization of Fabrication Parameters to Prepare Tea Catechin-Loaded Liposomes using Response Surface Methodology

[1]Jianjun Fang, [1]Rongfa Guan, [2]Chang Ri, [1]Mingqi Liu, [3]Xingqian Ye and [1]Jiaxin Jiang
[1]Zhejiang Provincial Engineering Laboratory of Quality Controlling Technology and Instrumentation for Marine Food, China Jiliang University, Hang-Zhou, 310018, China
[2]Hangzhou Test Institute of Quality, Hangzhou, Zhejiang, 310019, China
[3]Department of Food Science and Nutrition, Zhejiang University, Hangzhou, Zhejiang, 310029, China

Abstract: The purpose of this study was to optimize the formulation of tea catechin-loaded nano-liposomes using response surface methodology. Response surface methodology based on central composite rotatable design has been successfully used to model and optimize biochemical and biotechnological processes. The mass ratio of phosphatidylcholine and cholesterol (1-3), catechin concentration (3-5 mg/mL), pH values of phosphate buffer solution (6-7) and the volume ratio of organic phase and aqueous phase (2-4) were selected as independent variables with encapsulation efficiency and particle size as dependent variables. For each response, a second-order polynomial model was developed using multiple linear regression analysis. Applying a desirability function method the optimum parameters were: phosphatidylcholine to cholesterol mass ratio of 2.17, catechin concentration of 5 mg/mL, pH values of phosphate buffer solution of 6.62 and organic phase to aqueous phase volume ratio of 3.05. At this optimum point, particle size and encapsulation efficiency were found to be 220 nm and 60.18%, respectively. Furthermore, leakage ratio of nano-liposomes was used to determine the influence of storage period.

Keywords: Catechin, encapsulation efficiency, nano-liposomes, particle size, response surface methodology, stability

INTRODUCTION

Catechin, a major group of polyphenols extracted from green tea leaves, which exhibit a strong and powerful antioxidative, anti-obesity, hypolipidemic and anticarcinogenic activities and therapeutic potential in several chronic inflammatory diseases, including cancer (Anand *et al.*, 2008; Middleton *et al.*, 2000). However, its physicochemical properties generally result in poor chemical stability and lack of *in vivo* bioavailability (Cai *et al.*, 2002; Dvorakova *et al.*, 1999).

There has been considerable interest in liposomes (Rongen *et al.*, 1997), as they may be used for protection in food and pharmacy system (Felnerova *et al.*, 2004; Leserman, 2004; Torchilin, 2005). Liposomes are spherical vesicles with a diameter ranging from 20 nm to a few thousands nm, which are composed of a lipid bilayer with the hydrophobic chains of the lipids forming the bilayer and the polar head groups of the lipids orienting towards the extra vesicular solution and inner cavity (Edwards and Baeumner, 2006; Lorin *et al.*, 2004). The liposomes enhance the stability of the encapsulated material by protecting them from the environment (Mozafari *et al.*, 2008; Fathia *et al.*, 2012).

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques by analyzing the response surface contour to find optimal process parameters and using multiple quadratic regression equation to fit between the factors and the response function. RSM is a useful technology in developing processes and optimizing their performance (Myers and Montgomery, 1995; Raissi, 2009). Besides, response surface methodology has been successfully used to model and optimize biochemical and biotechnological processes related to food (Liyana-Pathirana and Shahidi, 2005; Pompeu *et al.*, 2009; Wang *et al.*, 2007).

The main objective of this study was to evaluate effect of the mole ratio of phosphatidylcholine and cholesterol, catechin concentration (w/v), pH and the ratio of organic phase and aqueous phase (v/v) on the Encapsulation Efficiency (EE) and Particle Size (PS) and to find out the optimal conditions for preparing the catechin nano-liposomes using RSM. Furthermore, leakage ratio of nano-liposomes was used to determine the influence of storage period.

Corresponding Author: Rongfa guan, Zhejiang Provincial Engineering Laboratory of Quality Controlling Technology and Instrumentation for Marine Food, China Jiliang University, Hang-Zhou, 310018, China

MATERIALS AND METHODS

Materials: Phosphatidylcholine (PC) was purchased from Beijing Shuangxuan Microbe Culture Medium Products Factory (Beijing, China). Cholesterol (CH) and Catechin were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Chloroform, diethyl ether was obtained from Hangzhou Jiachen Chemical Company. All chemicals were of reagent grade and used without further purification.

Preparation of liposomes: Catechin loaded liposomes were prepared by the reverse-phase evaporation (Szoka and Papahadjopoulos, 1978; Ming-Hui and Shi-Ying, 2007; Shah *et al*., 2012). Routinely, An appropriate amount of the lipid mixture of Soya Phosphatidylcholine (SPC) and Cholesterol (CS) was dissolved in a minimum amount of a mixed solvent of chloroform/ethanol (2:1, v/v) and then was dried to a thin film using a rotary evaporator under reduced pressure at a temp of 35°C and flask was rotated at 80 rpm. The residual solvent in the lipid film was removed by N_2 for 20 min and then was re-dissolved in ether. Then 3 mL of the aqueous phase solution containing phosphate-buffered saline (0.10 M, pH7, PBS) and catechin was added drop wise. The suspension was additionally sonicated in a bath-type sonicator for 10 min at 5°C. The mixture was again placed on a rotary evaporator and the organic solvent was removed under slightly reduced pressure, until the suspension became a gel, followed by continued evaporation under great vacuum. Eight mL of phosphate-buffered saline was added to the thin layer of round bottom flask to hydrate the layer and the suspension evaporated for an additional 25 min at 35°C to remove traces of solvent.

Table 1: Levels of factors used in CCRD

	Independent variable level				
Independent variables	Low	Center	High	Axial (-α)	Axial (+α)
PC:CH (w:w)	1	2	3	2.414	7.242
the concentration of catechin	3	4	5	7.242	12.07
pH values of phosphate buffer solution	6	6.5	7	14.484	16.898
Organic phase: aqueous phase (v:v)	2	3	4	4.828	9.656

Particle size: The particle size was measured by Mastersizer 2000 instrument (Malvern) (Sadowski *et al*., 2008), equipped with HydroMu dispersing unit (Malvern). Measurements were taken in the range between 0.1 and 1000 μm, under the following conditions: water refractive index 1.33 and general calculation model for irregular particles. The data obtained were averaged by software.

Encapsulation Efficiency determination (EE): The encapsulation efficiency was determined by centrifuge-UV method. Take lipsomes suspension (1.25mL) by spinning at 20000g for 1h using centrifuge, the catechin content of the supernatant was measured by UV spectrophotometry. The same suspension was ruptured using sufficient volume of ethanol and the total amount of Catechin was determined spectrophotometrically.

Table 2: Scheme of CCRD with the results of responses on four independent factors

	Independent variable				Response variable	
Run	pH values of phosphate buffer solution	PC:CH	Catechin concentration (w/v)	Organic phase: aqueous phase (v:v)	particle size (Y_1/nm)	EE (Y_2/%)
1	7	3	4	3	234	54
2	7	1	4	3	245	58.6
3	6.5	2	3	2	289	51
4	6.5	2	4	3	213	57.8
5	6.5	2	3	4	279	52
6	6	1	4	3	300	43.3
7	6	3	4	3	265	53.5
8	6.5	2	5	2	245	58
9	6.5	2	5	4	220	58.9
10	7	2	4	4	278	46.4
11	6.5	1	3	3	220	45
12	6.5	2	4	3	280	52.8
13	7	2	4	2	224	45.7
14	6	2	4	4	220	46
15	6.5	1	5	3	200	50
16	6.5	3	5	3	260	55.5
17	6.5	3	3	3	210	54
18	6	2	4	2	230	45.6
19	7	2	5	3	235	58.8
20	6.5	1	4	4	234	40.4
21	6.5	3	4	2	263	48
22	6.5	2	4	3	212	52.8
23	7	2	3	3	250	50
24	6.5	3	4	4	278	50.7
25	6.5	1	4	2	290	44.3
26	6	2	5	3	260	58.5
27	6	2	3	3	220	50

Encapsulation efficiency was calculated using Eq. (1):

$$EE\% = \frac{Q_t - Q_f}{Q_t} * 100 \quad (1)$$

where,
Q_f = The amount of free catechin
Q_t = The total amount of catechin present in 1.25 mL of nano-liposomes

Stability analysis: Catechin nano-liposomes were stored at 4°C in a refrigeratory. Take 0.50 mL samples at predetermine intervals. The leakage ratios of samples were calculated. Leakage ratios were calculated using Eq. (2):

$$L_O = \left(W_{EE} - W_{EE_t}\right) \Big/ W_{EE} \times 100\% \quad (2)$$

where,
W_{EE} = Encapsulation of preparation
W_{EE_t} = Encapsulation of a certain period of time

Experimental design and optimization: RSM as a generic method for optimization was applied to optimize the formulation of catechin liposomes. Based on the preliminary experiments and our previous studies, four formulation parameters which included PC: CH, catechin concentration, pH and the ratio of organic phase and aqueous phase were identified as key factors responsible for EE and particle size. In view of the feasibility of liposome preparation, the ranges of the four factors were determined as follows: PC: CH (1-3), catechin concentration (3-5, w/v), pH (6-7) and organic phase: aqueous phase (2-4, v/v) (Table 1). The experimental runs for CCRD were shown in Table 2. The response could be related to the selected variables by a second-order polynomial model. In this study, a second-order polynomial Eq. (2) was used to generate response surfaces:

$$\hat{Y}_i = b_0 + \sum_i b_i x_i + \sum_i b_{ii} x_i^2 + \sum_{i \neq j} b_{ij} x_i x_j \quad (3)$$

where,
$\hat{Y}_i$: Represents the predicted responses
x_i & x_j: The coded values of independent variables
b_0 : The intercept coefficient
b_i : The linear coefficients
b_{ii} : The squared coefficients
b_{ij} : The interaction coefficients (Zhang *et al.*, 2007)

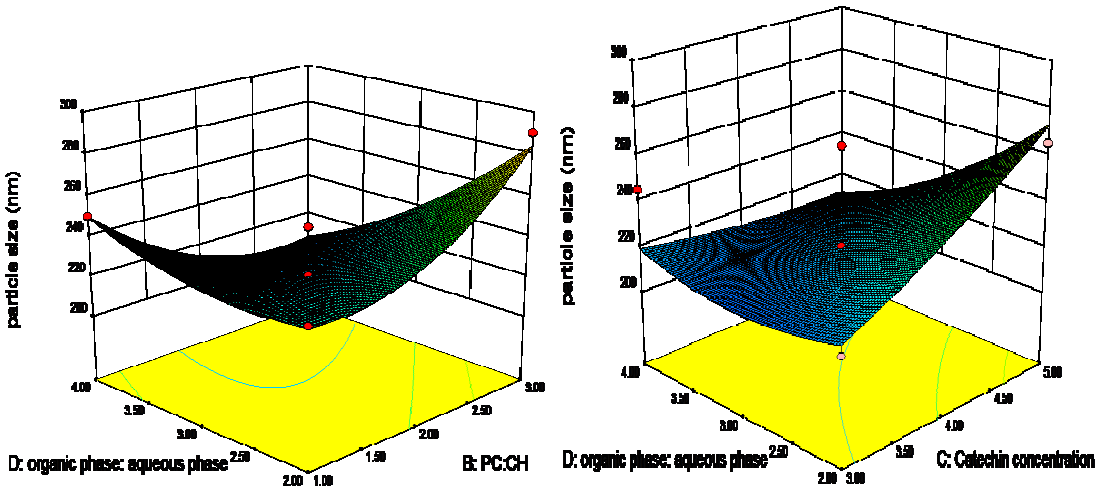
Statistical significance of the terms in the regression equations was examined. The significant terms in the model were found by Analysis of Variance (ANOVA) for each response. The adequacy of model was checked accounting for R^2 and adjusted-R^2. The desired goals for each variables and response were chosen. All the independents variables were kept within range while the responses were either maximized or minimized.

RESULTS AND DISCUSSION

Fitting the model: Table 2 showed the combined effects of phosphatidylcholine/cholesterol ratio, catechin concentration, pH and organic phase/aqueous phase ratio on PS and EE. The second-order polynomial

Table 3: ANOVA and regression coefficients of the second-order polynomial model for the response variables (actual values)

		PS (nm)			EE (%)		
Source	DF	Coefficient	S.S.	p-value	Coefficient	S.S.	p-value
Model	14	220.00	17282.00	<0.0001	52.96	555.52	<0.0001
Linear	1						
b1	1	4.420	234.080	0.3662	1.380	22.960	0.1000
b2	1	2.080	52.0800	0.6648	2.840	96.900	<0.050
b3	1	11.92	1704.08	<0.050	3.140	118.44	<0.050
b4	1	-14.92	2670.08	<0.010	0.150	0.2700	0.8481
Quadratic							
b11		33.62	6030.08	<0.010	-3.700	15.260	0.1703
b22		15.62	1302.08	<0.050	-3.330	59.110	0.0156
b33		2.370	30.0800	0.7414	2.220	26.300	0.0811
b44		7.880	330.750	0.2868	-4.620	113.67	0.0024
Interaction							
b12		-16.25	1056.25	0.0721	-3.700	54.76	0.0180
b13		10.00	400.000	0.2442	0.075	0.023	0.9559
b14		5.500	121.000	0.5116	0.075	0.022	0.9559
b23		-16.00	1024.00	0.0759	-0.880	3.060	<0.0001
b24		-23.50	2209.00	<0.050	1.650	10.89	0.2404
b34		-15.75	992.250	0.0779	-0.025	2.500E-003	0.9853
Residual	13		2612.67			69.91	
Total	27		21558.67			71448.72	
R^2		0.8687			0.8882		
Adj-R^2		0.6848			0.7317		
CV		6.5600			5.1700		



(a) (b)

Fig. 1: Response surface for the effect of independent variables on particle size of catechin nano-liposomes: Phosphatidylcholine to cholesterol and organic phase to aqueous phase (a, phosphatidylcholine to cholesterol ratio = 2.17 and organic phase to aqueous phase ratio = 3.05), catechin concentration and organic phase to aqueous phase (b, catechin concentration = 5 mg/mL and organic phase to aqueous phase = 3.05)

response surface model Eq. (3) was fitted to each of the response variables (Y_i). For the corresponding fitting of the explanatory models, the variations of particle size and encapsulation efficiency were analyzed. These analyses indicated that adding terms up to quadratic significantly improved the model (Table 1) and could be the most appropriate model for the two response variables.

Regression analysis and the Analysis of Variance (ANOVA) were used for fitting the model and to examine the statistical significance of the terms. The estimated regression coefficients for the response variables, along with the corresponding R^2, adj-R^2, Coefficient of Variation (CV), F-value and p-value of lack of fit, are shown in Table 3.

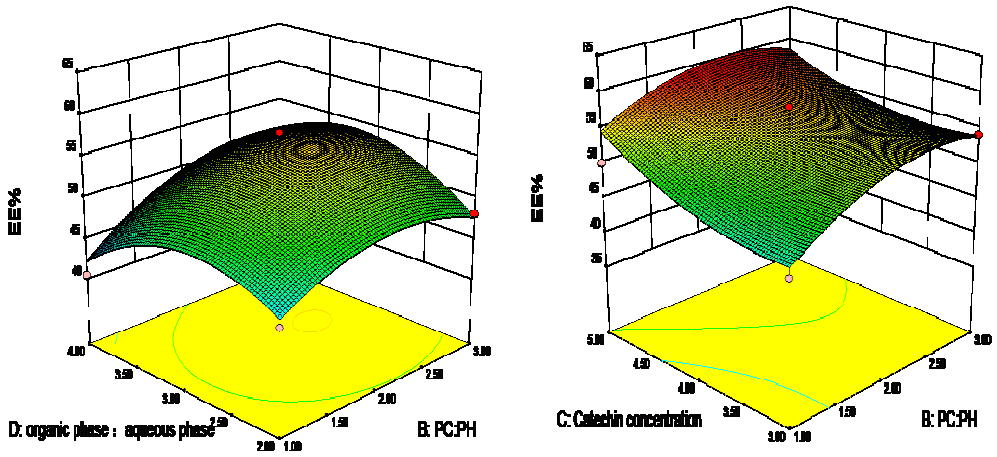
In addition, adj-R^2 and Coefficient of Variation (CV) were calculated to check the model adequacy. R^2 values for these response variables were higher than 0.80, indicating the regression models were suitable to explain the behavior, but a large value of R^2 does not always imply the adequacy of the model. Adding a variable to the model will always increase R^2, regardless of whether the additional variable is statistically significant or not. Thus, it is better to use an adj-R^2 to evaluate the model adequacy. The R^2 values were 0.8687 and 0.8882 for PS and EE respectively (Table 3). R^2 and adj-R^2 values for the model did not differ greatly; indicating non-significant terms have not been included in the model. As a general rule, a CV higher than 10% indicates that variation in the mean value is high and does not satisfactorily develop an adequate response model. The CV values for PS and EE were found to be 6.65 and 5.17 which represented a better reproducibility and reliability of the conducted experiments.

Particle size: Based on the sum of squares, the importance of the independent variables on yield could be ranked in the following order: organic phase to aqueous phase ratio> catechin concentration> pH> phosphatidylcholine to cholesterol ratio. Among the interaction terms, it shows that organic phase to aqueous phase ratio with phosphatidylcholine to cholesterol ratio were significant ($p<0.05$).

The effect of organic phase to aqueous phase ratio and phosphatidylcholine to cholesterol ratio on size is given in Fig. 1a. Wu *et al.* (2007) reported that particle size of papain decreases with decreasing phosphatidylcholine concentration due to that phospholipids constitute the liposome membrane and phosphatidylcholine concentration directly affects the particle size of the liposomes.

The variation of size with catechin concentration and organic phase: aqueous phase is presented in Fig. 1b. Increasing Catechin concentration or organic phase: aqueous phase does not have a significant effect on particle size.

It has also been cited that different drug concentrations have an effect on the particle size and dispersion of the liposomes (Zhang *et al.*, 2005). Similar trend has been reported for paclitaxe lmagnetic nanoparticle liposomes (Xiao and Wu, 2010), ferrous glycinate nanoliposomes (Ding *et al.*, 2011).



(a) (b)

Fig. 2: Response surface for the effect of independent variables on encapsulation efficiency of catechin nano-liposomes: Phosphatidylcholine to cholesterol and organic phase to aqueous phase (a, phosphatidylcholine to cholesterol ratio = 2.17 and organic phase to aqueous phase ratio = 3.05) and phosphatidylcholine to cholesterol ratio and catechin concentration (b, phosphatidylcholine to cholesterol ratio = 2.17 and catechin concentration = 5 mg/mL)

Table 4: Predicted optimum conditions of preparation of catechin nano-liposomes

Factor	Low	High	Optimum
Phosphatidylcholine: cholesterol	1	3	2.17
Catechin concentration	3	5	5
pH values of phosphate buffer solution	6	7	6.62
Organic phase: aqueous phase	2	4	3.05

Table 5: Predicted and experimental values of the responses obtained at optimum conditions

Response	Predicted value	Experimental value
PS (nm)	200	220±12
EE (%)	60.18	59%±0.38

Encapsulation efficiency: The results in Table 3 showed that the linear effect of phosphatidylcholine to cholesterol ratio and catechin concentration were significant ($p<0.05$) whereas pH and organic phase to aqueous phase ratio are not significant. The effect of independent variables on catechin nano-liposomes is shown in Fig. 2a, b. At higher catechin concentration, encapsulation efficiency is increased due to that more catechin was encapsuled into the vesicles. Besides increasing phosphatidylcholine to cholesterol ratio increased encapsulation efficiency. It may be that cholesterol can change the order of mobility of lecithin in the lipid bilayer, thus reinforcing the membrane stability (Niu *et al.*, 2011). Similar results were observed in the studies by Kontogiannopoulos *et al.* (2011) and Xiong *et al.* (2009).

Optimization: Our optimization experiments were designed to find the maximum encapsulation efficiency and minimum particle size of catechin nanoliposomes.

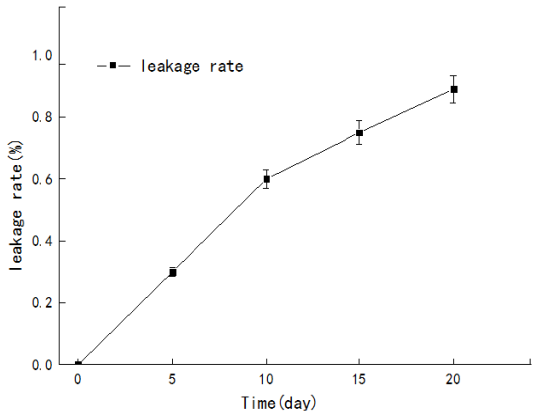


Fig. 3: Storage stability of nano-liposomes
Data reported are the mean values±standard variation of three replications

Phosphatidylcholine/ cholesterol ratio, catechin concentration, pH and organic phase/aqueous phase ratio were selected in the range of 1-3, 3-5, 6-7 and 2-4 mg/mL, respectively. Table 5 shows the conditions given the lowest value of particle size (224 nm) with highest encapsulation efficiency (60.18%).These are: phosphatidylcholine to cholesterol ratio of 2.17, catechin concentration of 5 mg/mL, pH of 6.62 and organic phase to aqueous phase ratio of 3.05 (Table 4).

Stability: The catechin nano-liposomes were subjected to storage stability study for the period of 20 days. The storage stability of catechin nano-liposomes composed of PC: CH ratio of 2.17, catechin concentration of 5 mg/mL, pH of 6.62 and organic phase to aqueous phase ratio of 3.05 at 4°C is presented in Fig. 3. As it shows, the leakage ratio of catechin nano-liposomes tended to

increase with increasing storage period. This observation suggests that the leakage of catechin nano-liposomes might be attributed to hydrolyzation and degradation of bilayer membranes and/or vesicle fusion/aggregation (Flatena *et al.*, 2008; Hincha, 2003; Wang *et al.*, 2011).

CONCLUSION

The effect of cholesterol to phosphatidylcholine ratio, catechin concentration, pH and aqueous phase to organic phase ratio on preparing catechin nano-lipsomes were studied. Second-order polynomial models were obtained for predicting particle size and encapsulation efficiency. While increasing the cholesterol to phosphatidylcholine ratio increased the particle size and encapsulation efficiency. Numerical optimization determined the optimum preparation conditions, which were phosphatidylcholine to cholesterol ratio of 2.17, catechin concentration of 5 mg/mL, pH of 6.62 and organic phase to aqueous phase ratio of 3.05. Furthermore, leakage ratio of nano-liposomes was tested for the period of 20 days. The catechin nano-liposomes showed an acceptable stability.

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