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Optimization on preparation conditions of *Rehmannia glutinosa* polysaccharide liposome and its immunological activity

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ABSTRACT

The aim of this study was to investigate the optimizing preparation conditions and immunological enhancement activity of *Rehmannia glutinosa* polysaccharide liposome (RGPL). RGPL was prepared using the reverse-phase evaporation method and optimized using the response surface methodology. The immunological enhancement activity of RGPL on splenocyte proliferation was measured. These results showed that the optimum preparation conditions were: a soybean phosphatide to cholesterol ratio of 8:1, a chloroform to methanol ratio of 3:5, a soybean phosphatide to tween 80 ratio of 10:1, a temperature (°C) of 66 °C and an entrapment efficiency of 72.753 ± 0.318%. In a single stimulation of drugs, RGPL could significantly promote splenocyte proliferation, specifically at 200 μg mL^{-1}. Moreover, in a synergistic stimulation of drugs with LPS or PHA, a significant difference was obtained between the RGPL and RGP at 100–400 μg mL^{-1}, which indicated that the immunological enhancement of RGP was significantly improved after encapsulation with the liposome.

1. Introduction

Rehmannia glutinosa, a therapeutic Scrophulariaceae herb, has been used for different medical purposes as a traditional Chinese herbal medicine for thousands of years. *R. glutinosa* polysaccharide (RGP) is one of the major active components of *R. glutinosa*. Thus far, it has been reported that RGP has various pharmacological properties, such as strengthening hematopoiesis (Liu, Miao, & Gao, 2008; Miao, Wang, & Sun, 2007), antioxidation (Miao, Sun, & Fang, 2002), anti-mutagenesis (Xu, Liang, & Gao, 2009), enhancing memory ability and maintaining caducity (Cui, Hou, Yan, & Chang, 2003). Furthermore, several studies have described that RGP could strengthen immunity (Kang & Kim, 2011; Zhang, Li, & Jia, 2008; Zhang, Meng, & Guo, 2010). However, RGP has many disadvantages when directly used for clinical purposes, such as a fast-metabolism, short action time, and non-focused action scope, among others. Thus, it is necessary to increase the bioavailability of RGP in clinical applications.

Liposomes are artificial microscopic vesicles with an aqueous core enclosed in one or more phospholipid bilayers (Sharma & Sharma, 1997). One or more active pharmaceutical ingredients can be encapsulated in its aqueous core and/or the membrane (Ohnishi et al., 2013). Liposomes, as novel drug delivery systems and promising adjuvants, have been the subject of intensive investigations for the past few decades. They can transport drugs for site-specific delivery to enhance efficacy and bioavailability, sustain slow-release, decrease adverse reactions and improve patient compliance (Date, Joshi, & Patravale, 2007). There have already been more than 10 FDA-approved liposomal drugs (Wagner, Dullaart, Bock, & Zweck, 2006). In addition, it has been reported that liposomes have been extensively used as an adjuvant for vaccine antigens against bacterial (Lachman, Ozpolat, & Rao, 1996; Sinha & Khuller, 1997) and viral (Ambrosch et al., 1997; Okada et al., 1997) infections and tumors (Kwak et al., 1998; Okamoto et al., 1997).

Response surface methodology (RSM) is a collection of statistical and mathematical methods that are useful for planning experiments, determining complex quantitative relationships between parameters and their responses and identifying response optimizing factor combination (Cai, Gu, & Tang, 2007; Lee, Yusof, Hamid, & Baharin, 2006). It is an effective and powerful statistical method for optimization of various complex processes (Gan & Latiff, 2011; Ye & Jiang, 2011) and has advantages in terms of reducing the

Abbreviations: RGPL, *Rehmannia glutinosa* polysaccharide liposome; RGP, *Rehmannia glutinosa* polysaccharide; RSM, response surface methodology; PHA, phytohemagglutinin; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; EE, entrapment efficiency; BBD, Box–Behnken design; BL, blank liposome.

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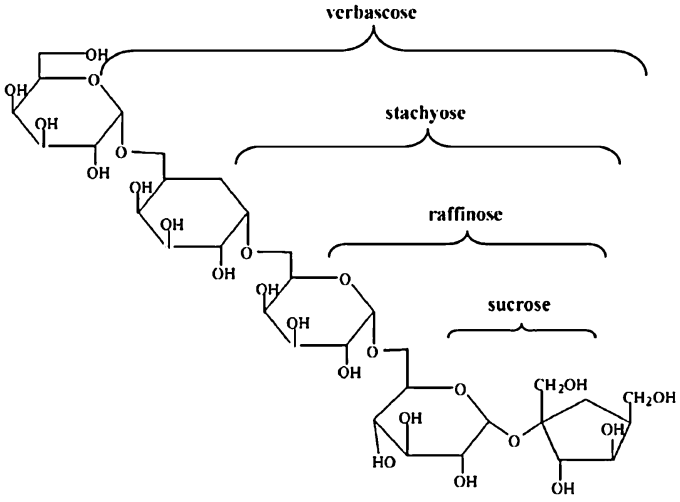
number of experimental trials required to evaluate multiple parameters and their interactions (Domingos, Saad, Wilhelm, & Ramos, 2008). Box–Behnken design (BBD), one of the most common designs of the principal RSM, has been widely applied in the optimization of chemical and physical processes due to its rational design and excellent interpretation of experiments (Donga, Xie, Wang, Zhana, & Yao, 2009; Ferreira et al., 2007).

In this study, the RGP was encapsulated in the liposome using the reverse-phase evaporation method, and the preparation conditions of RGPL were optimized with RSM. Next, the immunological enhancement activity of RGPL on splenocyte proliferation was measured. The objective of this study was to improve the immunological enhancement activity of RGP via the combination of the advantages of the liposome with the immunological function of RGP.

2. Materials and methods

2.1. Materials

R. glutinosa polysaccharide (with a purity of 98% HPLC, Mol.wt: 3.57×10^4, basic structure was purchased from Shanxi Ciyuan Biotechnology Co. Ltd. Soybean phospholipid (No. 20120304) was manufactured by Shanghai Taiwei Pharmaceutical Co., Ltd. Cholesterol (No. 20120908) was purchased from Tianjin Bodi Chemical Technology Co., Ltd. Triton X-100 (No. 9002931) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. Vanillin (No. 121335) was obtained from Tianjin Kemiou Chemicals R & D Center. RPMI-1640 (GIBCO) supplemented with benzylpenicillin 100 IU mL^{-1}, streptomycin 100 IU mL^{-1} and 10% fetal bovine serum. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Amresco Co.) was dissolved into 5 mg mL^{-1} with calcium and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.4). Dimethyl sulfoxide (DMSO) was purchased from Zhengxing Institute of Chemical Engineering in Suzhou, China.



2.2. Optimization on the preparation methods of RGPLs

2.2.1. Preparation of RGPLs

RGPL was prepared using the reverse-phase evaporation method. Briefly, soybean phospholipid, cholesterol and Tween-80 were dissolved in a solution with chloroform (5 mL) and methanol (5 mL) with the help of ultrasonic cleaner. Organic solvent was later removed using a rotary vacuum evaporation under lipid transition temperature. After the lipid film was formed, ethyl ether was added to dissolve it and RGP (2 mg mL^{-1}) was injected into the solution. The resulting mixture was homogenized using an ultrasonic cleaner in ice water to form a stabile W/O emulsion. The solution was evaporated to form a colloid using a vacuum and PBS was added to hydrate for an additional 15 min. Next, the resulting mixture was homogenized using an ultrasonic cell disintegrator (JY92-II DN, Xinzhi Bio-technology and Science Inc.) for 20 min to form the uniform liposome. Ultimately, the solution was successively filtered using 0.45 μm and 0.22 μm millipore membrane.

Table 1
Levels and code of variables chosen for Box–Behnken design.

Factors	Code	Levels and range		
		−1	0	1
A (Soybean phosphatide to cholesterol ratio (w/w))	X_1	5	8	10
B (Chloroform to methanol ratio (v/v))	X_2	2	4	6
C (Soybean phosphatide to tween 80 ratio (w/w))	X_3	16	10	8
D (Temperature (°C))	X_4	50	60	70

2.2.2. Entrapment efficiency (EE) and drug-loading rate of RGPL array

Next, 0.1 mL of RGPL was added in a 10 mL centrifuge tube and mixed with 0.1 mL of protamine solution (Sigma, P4380, 10 mg mL^{-1}). After 3 min, physiological saline (3.0 mL) was added. After adequate mixing, this suspension was centrifuged at 4000 r min^{-1} at room temperature for 30 min. All of the supernatant was taken from the centrifuge tube and 2 mL was used to assay the content of RGP using the vitriol–phenol method, and the content of RGP was referred to as the content of free drug. The precipitation remaining in the centrifuge tube was dissolved with 0.6 mL of Triton X-100 and 2.6 mL of physiological saline. Next, 2 mL of the solution was used to assay the content of RGP using the vitriol–phenol method, and this solution was called the content of encapsulated drug. The formula to calculate the liposome encapsulation efficiency was $EE\% = (1 - C_f/C_t)$, C_f is the content of free drug, C_t is the total content of drug (Guo, Cheng, & Lin, 2010). The formula to calculate the liposome drug-loading rate was $W\% = [(W_T - W_F)/W_P] \times 100\%$, W_T is the total content of drug, W_F is the content of free drug, and W_P is the total content of soybean phospholipid and cholesterol.

2.3. Single-factor test of RGPL preparation

Preparation of the RGPL was the same as described in Section 2.2.1. The effects of four single factors (ratio of soybean phospholipid to cholesterol, ratio of lipid to tween 80, ratio of chloroform to methanol and rotary evaporation temperature) on the EE and drug-loading rate of RGPL were observed.

2.4. Optimization of RGPL preparative condition

On the basis of single factor investigation, the preparation parameters were optimized using RSM. A three level-four variable BBD was adopted in this study, and 27 experiments were required to establish optimum conditions for the preparation of RGPL. The parameters and their levels were presented in Table 1, and EE was selected as the response (Y). The behavior of the system was explained using the following quadratic equation:

$$Y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} b_{ij} x_i x_j \quad (1)$$

where Y is the dependent variable (EE in real value); b_0, b_i, b_{ii} and b_{ij} are coefficients estimated by the model; and X_i and X_j are levels of the independent variables. They represent the linear, quadratic

and cross product effects of the X_1, X_2, X_3 and X_4 factors on the response, respectively.

Experimental data were fitted to the Design-Expert program (Version 8.0.6, Stat-Ease Inc., Minneapolis, MN, USA), and the tri-dimensional response surfaces and contour plots were also generated. These response surfaces and contour plots could visualize the relationship between the response and experimental levels of each factor and deduce parameter interactions (Triveni, Shamala, & Rastogi, 2001). Subsequently, the optimum condition was verified by performing three additional confirmation experiments under these conditions.

2.5. Immunological activity of RGPL in vitro

Splenic lymphocytes were harvested as previously reported (Huang et al., 2013), with some modifications. Briefly, the spleens were sterilely harvested from the ICR mouse and gently ground by pressing against a stainless steel sieve (200 mesh). After the red blood cells were disrupted using ACK lysis buffer (Sigma), the splenocytes were washed twice. The resulting pellet was re-suspended and diluted to 2.5×10^6 mL^{-1} with RPMI-1640 in fetal bovine serum after the cell viability was assessed using trypan blue exclusion. The solution was divided into two parts: one part was added with phytohemagglutinin (PHA, Sigma, No. L-8754, 0.1 mg mL^{-1}) or lipopolysaccharide (LPS, Sigma, No. L2880, 0.05 mg mL^{-1}) and incubated into 96-well culture plates with 100 μL per well. Next, RGPL, RGP and blank liposome (BL) at a series of concentrations were added, in the cell control group and PHA or LPS control group, RPMI-1640 medium and PHA or LPS, respectively, at 100 μL per well, four wells for each concentration. The final concentration of PHA or LPS reached to 10 μg mL^{-1} or 5 μg mL^{-1}. The plates were then incubated in a humid atmosphere with 5% CO_2 (Thermo) at 37 °C for 48 h. Next, 30 μL of MTT (5 mg mL^{-1}) was added into each well at 4 h prior to the end of the incubation. Next, the plates were centrifuged at 1000 × *g* for 10 min at room temperature. The supernatant was removed carefully and 100 μL of DMSO was added into each well. The plates were shaken for 10 min to completely dissolve the crystals. The absorbance of the cells in each well was measured using the microliter enzyme-linked immunosorbent assay reader (Model RT-6000, Leidu Co., Ltd. Shenzhen City) at a wavelength of 570 nm (A_{570} value) as an index of lymphocyte proliferation (Fan et al., 2012).

2.6. Statistical analysis

Data of the optimization of RGPL preparation were analyzed using the Design-Expert program. The second-order polynomial equation, ANOVA of the quadratic regression model, and optimal conditions were obtained. Data of the immunological activity experiment were expressed as the mean ± standard errors (S.E.). The Duncan and LSD's multiple range tests were used to determine the difference among groups. *P*-values of less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Effect of a single factor on the encapsulation efficiency and drug-loading rate of RGPL

3.1.1. Effect of the ratio of soybean phospholipid to cholesterol on the encapsulation efficiency and drug-loading rate of RGPL

The ratio of the soybean phospholipid to cholesterol was changed to 10:1, 8:1, 5:1, 4:1 and 2:1, and the other preparation conditions of RGPL (the usage amount of soybean phospholipid, ratio of lipid to drug, ratio of lipid to tween 80, ratio of lipid to chloroform and methanol, ratio of chloroform to methanol and rotary evaporation temperature) were controlled at the same level. According to the results shown in Fig. 1A, it is better that the soybean phospholipid:cholesterol ratio was chosen at 8:1 in later experiments.

3.1.2. Effect of the ratio of lipid to tween 80 on the encapsulation efficiency and drug-loading rate of RGPL

The ratio of lipid to tween 80 was changed to 5:1, 8:1, 10:1, 16:1 and 20:1 and the other preparation conditions of RGPL (the usage amount of soybean phospholipid, ratio of lipid to drug, ratio of soybean phospholipid to cholesterol, ratio of lipid to chloroform and methanol, ratio of chloroform to methanol and rotary evaporation temperature) were controlled at the same level. According to the results shown in Fig. 1B, it is better that the lipid:tween 80 ratio was chosen at 10:1 in later experiments.

3.1.3. Effect of the ratio of chloroform to methanol on the encapsulation efficiency and drug-loading rate of RGPL

The ratio of chloroform to methanol was changed to 8:0, 2:6, 4:4, 6:2 and 0:8 and the other preparation conditions of RGPL (the usage amount of soybean phospholipid, ratio of lipid to drug, ratio of soybean phospholipid to cholesterol, ratio of lipid to tween 80, ratio of lipid to chloroform and methanol and rotary evaporation temperature) were controlled at the same level. According to the results shown in Fig. 1C, it is better that the chloroform:methanol ratio was chosen at 4:4 in later experiments.

3.1.4. Effect of the rotary evaporation temperature on the encapsulation efficiency and drug-loading rate of RGPL

The rotary evaporation temperature was changed to 30, 40, 50, 60 and 70 °C and the other preparation conditions of RGPL (the usage amount of soybean phospholipid, ratio of lipid to drug, ratio of soybean phospholipid to cholesterol, ratio of lipid to tween 80, ratio of lipid to chloroform and methanol and ratio of chloroform to methanol) were controlled at the same level. According to the results shown in Fig. 1D, it is better that the rotary evaporation temperature was chosen at 60 °C in later experiments.

3.2. Statistical analysis and model fitting

RSM is an effective statistical technique for optimizing complex processes because it allows a more efficient and easier arrangement and interpretation of the experiments compared with other methods (Gan & Latiff, 2011; Gan, Manaf, & Latiff, 2010). There was a 27-run BBD with four factors and three levels for fitting a second-order response surface to optimize the preparation conditions. The experimental conditions and the EE of RGPL according to the factorial design are shown in Table 2. These results also showed that the EE ranged from 28.35% to 73.11%. The maximum EE value (73.11%) was found in conditions of X_1 = 8:1, X_2 = 4:4, X_3 = 10:1 and X_4 = 60 °C. The values of the regression coefficients were calculated, and the response variable and test variables were related by the following second-order polynomial equation:

$$Y = 71.01 + 1.58X_1 - 4.69X_2 + 4.56X_3 + 2.53X_4 - 6.14X_1X_4 + 8.11X_2X_3 - 9.30X_2X_4 - 7.88X_1^2 - 10.13X_3^2 - 16.85X_3^2 - 6.70X_4^2 \quad (2)$$

The statistical significance of the regression model was confirmed using the *F*-test and *P*-value, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Table 3. The determination coefficient (R^2 = 0.9649), shown using ANOVA of the quadratic regression model indicated that 96.49% of the variability in the response of EE could be explained using the model Eq. (2), which indicated that the model was adequate to predict within the range of experimental variables. The *P*-values

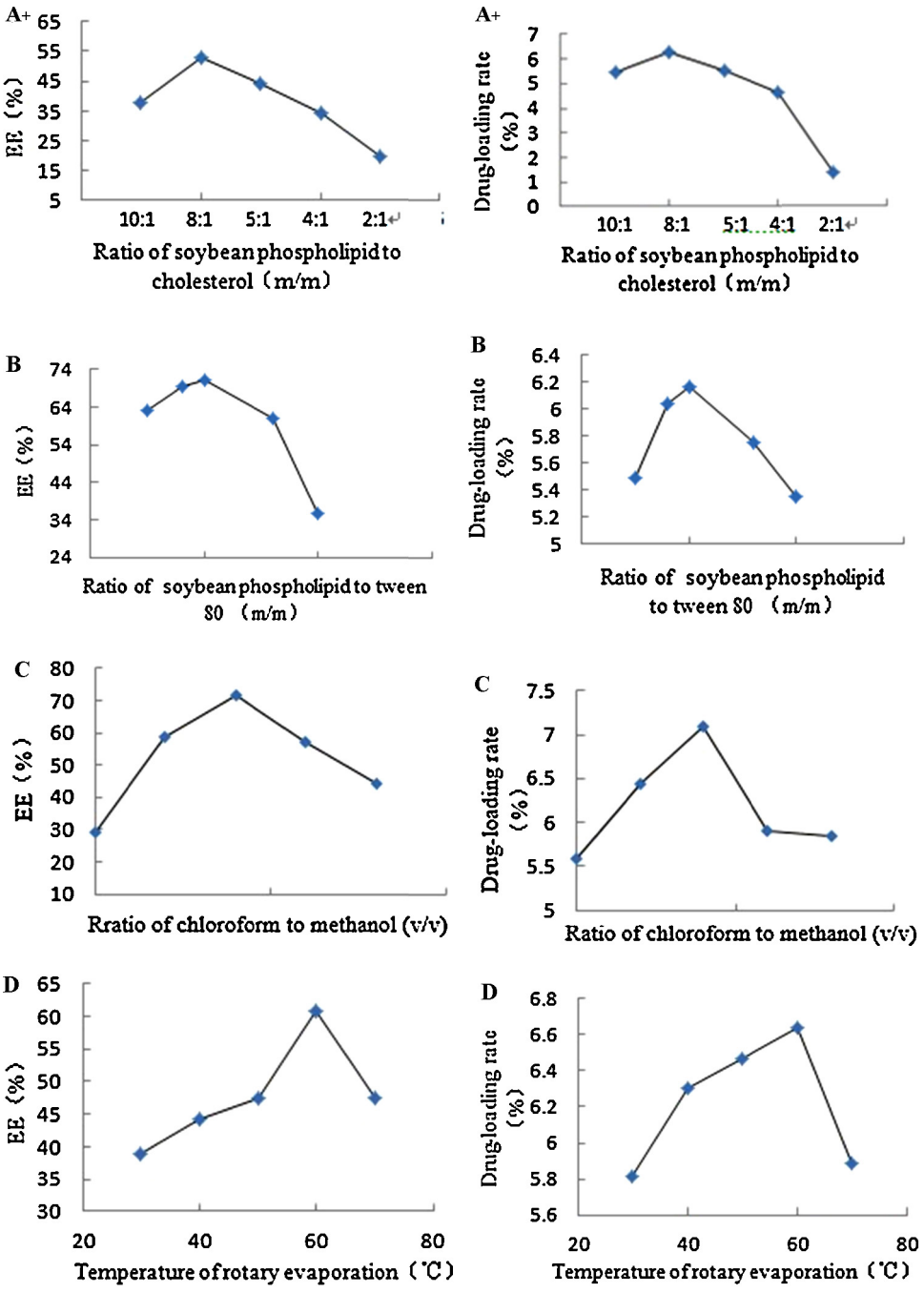


Fig. 1. The effect of different single factors on the EE and drug-loading rate of RGPL.

were used as a tool to confirm the significance of each coefficient. Furthermore, the smaller the *P*-value, the more significant the corresponding coefficient (Guo, Zou, & Sun, 2010). In this table, the linear coefficients (X_2, X_3, X_4), a quadratic term coefficient (X_1^2, X_2^2, X_3^2, X_4^2) and the interaction coefficient ($X_1 \times X_4$, $X_2 \times X_3$, $X_2 \times X_4$) were significant ($P < 0.02$). The other term coefficients (X_1) were not significant ($P > 0.05$). The full model filled Eq. (2) generated three-dimensional and contour plots to predict the relationships between the independent variables and dependent variables.

3.3. Optimization of RGPL preparation conditions

Results of the EE of RGPL affected by the soybean phosphatide to cholesterol ratio (w/w), chloroform to methanol ratio (v/v), soybean phosphatide to tween 80 ratio (w/w) and temperature (°C) were presented in Fig. 2. These types of plots showed the effects of two factors on the response at a time and the other factors were maintained at the zero level.

The maximum value predicted by the surface was confined in the smallest ellipse in the contour diagram. The elliptical contours were obtained when there was a perfect interaction between the independent variables (Chen et al., 2013; Yin, You, & Jiang, 2011).

The 3-D response surface plot and contour plot in Fig. 2A, which made EE as a function of soybean phosphatide to cholesterol ratio and temperature at fixed chloroform to methanol ratio (4:4) and soybean phosphatide to tween 80 ratio (10:1), indicated that EE gradually increased with an increase in the soybean phosphatide to cholesterol ratio from 5:1 to 10:1. In addition, EE

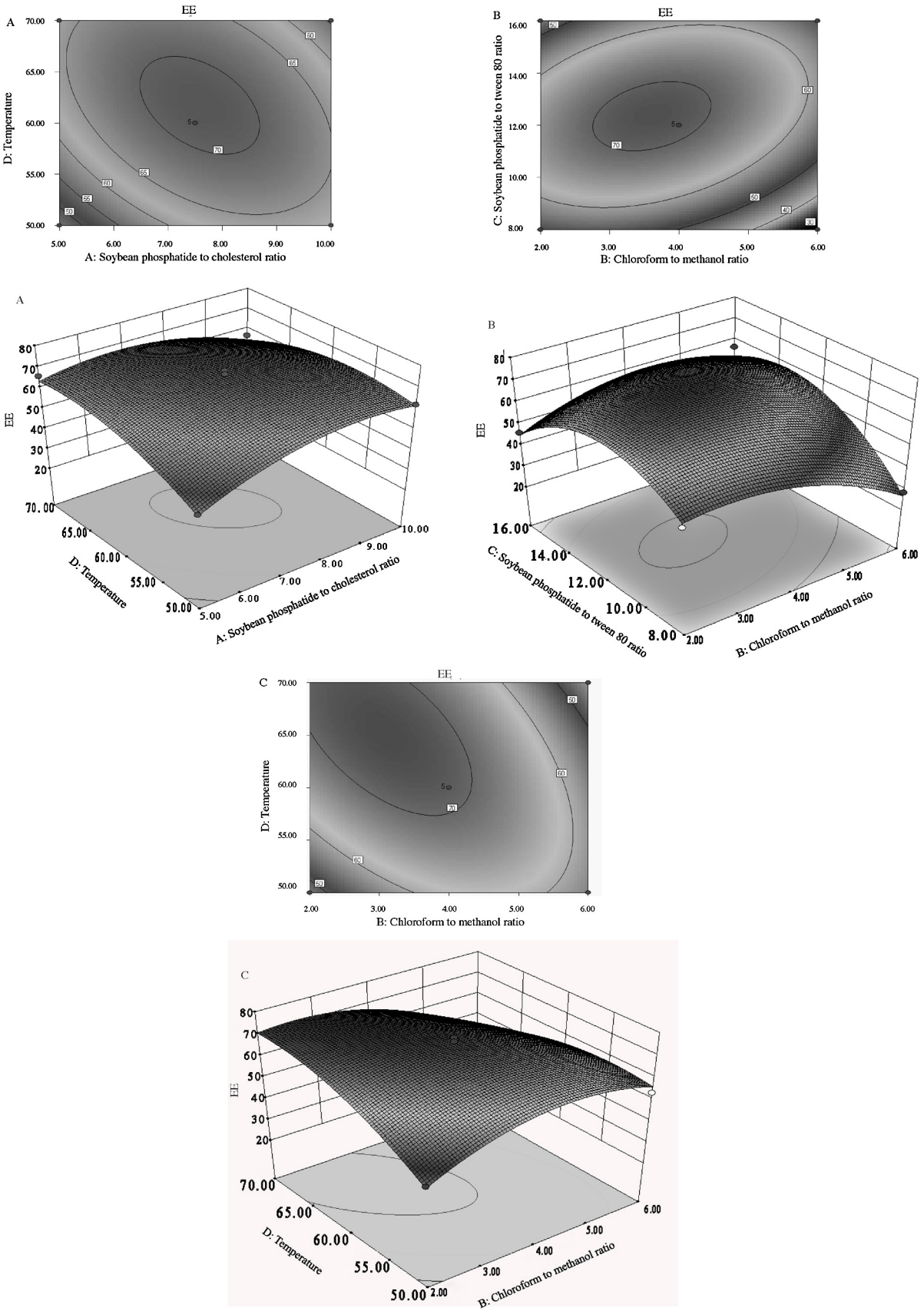


Fig. 2. Contour plots and response surface (3-D) showing the effects of soybean phosphatide to cholesterol ratio, chloroform to methanol ratio, soybean phosphatide to tween 80 ratio and temperature on the response Y.

Table 2
The design and results of Box–Behnken experiments.

No.	Levels of independent factors				Response: EE (%)	
	X_1	X_2	X_3	X_4	Predicted acquired ER	Practical acquired ER
1	8:1	4:4	10:1	60	71.011	71.459
2	8:1	4:4	8:1	70	45.433	47.68084
3	5:1	4:4	10:1	70	63.505	65.85889
4	10:1	4:4	10:1	70	54.399	56.62181
5	5:1	4:4	10:1	50	46.168	46.67987
6	8:1	6:2	16:1	60	52.002	56.59284
7	10:1	6:2	10:1	60	49.886	50.38208
8	8:1	2:6	16:1	60	45.174	46.24039
9	8:1	2:6	10:1	50	47.033	47.77342
10	10:1	2:6	10:1	60	59.268	59.31855
11	10:1	4:4	8:1	60	43.308	41.66793
12	8:1	6:2	10:1	70	42.713	39.08066
13	8:1	4:4	16:1	50	49.486	47.43973
14	8:1	6:2	8:1	60	26.677	28.34542
15	8:1	2:6	8:1	60	52.270	50.4131
16	10:1	4:4	10:1	50	61.613	61.99366
17	5:1	4:4	8:1	60	40.138	36.41295
18	5:1	4:4	16:1	60	49.253	50.34287
19	8:1	4:4	10:1	60	71.011	70.2981
20	8:1	4:4	10:1	60	71.011	71.383
21	8:1	4:4	8:1	50	40.372	43.67716
22	8:1	4:4	16:1	70	54.548	51.35621
23	5:1	6:2	10:1	60	46.716	46.48529
24	8:1	6:2	10:1	50	56.249	53.35673
25	10:1	4:4	16:1	60	52.422	50.91322
26	8:1	4:4	10:1	60	71.011	68.80622
27	8:1	4:4	10:1	60	71.011	73.10744

increased with an increase in temperature from 50 to 60 °C and then reached a plateau region where the EE was slightly changed after 60 °C.

The 3-D response surface plot and the contour plot at varying chloroform to methanol ratios and soybean phosphatide to tween 80 ratios at fixed soybean phosphatide to cholesterol ratio (10:1) and temperature (60 °C) (Fig. 2B). In addition, the EE increased rapidly within the chloroform to methanol ratio from 2:6 to 4:4, but when greater than 4:4, the EE began to decrease. Compared with the soybean phosphatide to tween 80 ratio, and a similar tendency was obtained. From 8:1 to 10:1, EE increased rapidly; afterwards, it decreased.

The 3-D response surface plot and the contour plot were developed for the EE with varying chloroform to methanol ratios and temperatures at fixed soybean phosphatide to cholesterol ratio (10:1) and soybean phosphatide to tween 80 ratio (10:1) (in Fig. 2C). This indicated that the maximum EE could be achieved when the chloroform to methanol ratio and temperature were within the level range from 4:4 to 6:2 and nearly 60 °C.

It could be concluded that the optimal preparation conditions for RGPL are soybean phosphatide to cholesterol ratio of 7.66:1, chloroform to methanol ratio of 3.01:4.99, soybean phosphatide to tween 80 ratio 10.06:1 and temperature of 65.92 °C (from Fig. 2). Among the four preparation parameters that have been studied, soybean phosphatide to tween 80 was the most significant factor that affected the EE, followed by the chloroform to methanol ratio and temperature and soybean phosphatide to cholesterol ratio according to the regression coefficients significance of the quadratic polynomial model (Table 4) and gradient of slope in the 3-D response surface plot (Fig. 2).

3.4. Verification of the predictive mode

The suitability of the model equation for predicting the optimum response values was tested using the selected optimal conditions. The maximum predicted EE and experimental EE are given in Table 4. To confirm the validity of the suggested mathematical model, additional experiments to re-confirm were performed under the modified optimal conditions: a soybean phosphatide to cholesterol ratio of 8:1, a chloroform to methanol ratio of 3:5, a soybean phosphatide to tween 80 ratio of 10:1 and a temperature (°C) of 66 °C.

The set of conditions were determined to be optimum by the RSM and were also validated experimentally and predicted the values of the responses using the model equation. A mean EE value of 72.753 ± 0.318% (N = 3) was obtained from practical experiments and demonstrated the validation of the RSM model, which indicated that the model was adequate for the preparation process (Table 4).

On the basis of the preliminary single-factor experiments, four factors (soybean phosphatide to cholesterol ratio (w/w), chloroform to methanol ratio (v/v), soybean phosphatide to tween 80 ratio (w/w) and temperature (°C)) were identified as key factors responsible for EE. RSM has been found to be accurate and economical during optimization of various industrial processes (Deepak et al., 2008; Gan & Latiff, 2011). It enables an evaluation of the effects of many factors and their interactions on response variables in a less laborious and time-consuming manner. Under optimal conditions, RGPL was prepared with high EE (72.753 ± 0.318%). The immunological activity of RGPL was further investigated.

Table 3
ANOVA for response surface quadratic model.

Source	Sum of squares	df	Mean square	F value	Prob > F
Model	3356.189	11	305.108	37.445	<0.0001 significant
A	26.61737	1	26.617	3.267	0.0908
B	185.472	1	185.472	22.762	0.0002
C	249.230	1	249.230	30.587	<0.0001
D	63.424	1	63.424	7.784	0.0137
AD	150.686	1	150.686	18.493	0.0006
BC	262.766	1	262.766	32.248	<0.0001
BD	211.446	1	211.445	25.950	0.0001
A^2	373.792	1	373.792	45.874	<0.0001
B^2	541.973		541.973	66.514	<0.0001
C^2	1741.082	1	1741.082	213.677	<0.0001
D^2	262.481	1	262.481	32.213	<0.0001
Residual	122.223	15	8.148		
Lack of fit	112.120	11	10.193	4.035	0.0950 not significant
Pure error	10.103	4	2.526		
Cor total	3478.412	26			

R^2 = 0.9649; R^2_{Adj} = 0.9391; R^2_{Pred} = 0.8221.

Table 4
Predicted and experimental values of the responses at optimum conditions.

	Soybean phosphatide to cholesterol ratio (w/w)	Chloroform to methanol ratio (v/v)	Soybean phosphatide to tween 80 ratio (w/w)	Temperature (°C)	EE (%)
Optimum conditions	7.66:1	3.01:4.99	10.06:1	65.92	72.861 (predicted)
Modified conditions	8:1	3:5	10:1	66	72.753 ± 0.318 (actual)

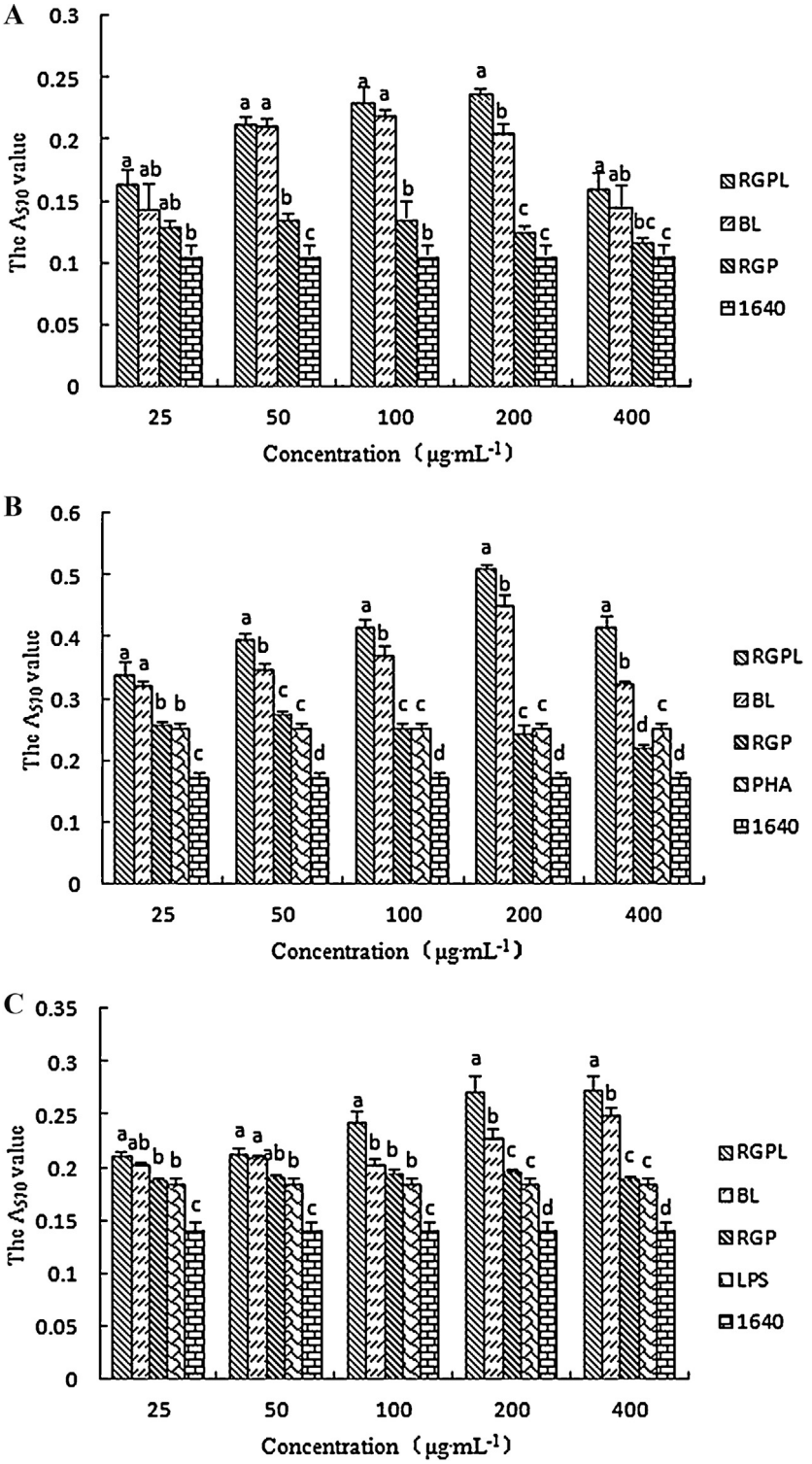


Fig. 3. (A) Effects of splenic lymphocyte proliferation in single stimulation of drugs (A_{570}). $^{a-c}$Bars in the figure without the same superscripts differ significantly ($P < 0.05$). (B) Effects of splenic lymphocyte proliferation in synergistical stimulation of drugs with PHA (A_{570}). $^{a-d}$Bars in the figure without the same superscripts differ significantly ($P < 0.05$). (C) Effects of splenic lymphocyte proliferation in synergistical stimulation of drugs with LPS (A_{570}). $^{a-d}$Bars in the figure without the same superscripts differ significantly ($P < 0.05$).

3.5. Effect of RGPL on splenic lymphocyte proliferation in vitro

The spleen represents an important and convenient lymphoid organ to study the complex interplay between cells of the innate and adaptive immune response (Richard et al., 2005). The splenic lymphocyte is important for the immunological response in the organism and proliferation of splenic lymphocyte is the most immediate index that reflects organic immunity (Pan, Liu, & Wang, 2011). To investigate the role of RGPL on immunological enhancement, the effect on splenic lymphocyte proliferation was examined.

In a single stimulation of RGPL, RGP or BL on splenic lymphocyte, the A_{570} values of RGPL were higher compared with those of RGP and BL under the same concentration ($P < 0.05$) (Fig. 3A). The A_{570} values of RGPL varied with a change in drug concentration. From 25 $\mu g\,mL^{-1}$ to 400 $\mu g\,mL^{-1}$, the A_{570} values initially turned higher but lowered after 100 $\mu g\,mL^{-1}$; the highest A_{570} value was at 100 $\mu g\,mL^{-1}$.

The A_{570} values of RGPL (25–400 $\mu g\,mL^{-1}$) were significantly higher than those of the RGP and BL groups under the same concentration after synergistic stimulation with PHA ($P < 0.05$) (Fig. 3B). The A_{570} values gradually became higher from 25 $\mu g\,mL^{-1}$ to 200 $\mu g\,mL^{-1}$ but lowered after 200 $\mu g\,mL^{-1}$. The highest value was obtained at 200 $\mu g\,mL^{-1}$.

The A_{570} values of RGPL (25–400 $\mu g\,mL^{-1}$) were significantly higher than those of the RGP and BL groups under the same concentration after synergistic stimulation with LPS ($P < 0.05$) (Fig. 3C). The A_{570} values gradually became higher from 25 $\mu g\,mL^{-1}$ to 400 $\mu g\,mL^{-1}$ and reached the highest value at the highest concentration (400 $\mu g\,mL^{-1}$).

The RGPL, RGP and BL can all stimulate splenic lymphocyte proliferation in vitro (Fig. 3A). Among them, RGPL stimulation on lymphocyte cells was the most significant under a concentration between 25 $\mu g\,mL^{-1}$ and 400 $\mu g\,mL^{-1}$. This finding indicated that stimulation on splenic lymphocyte proliferation of RGP was significantly improved after encapsulation with liposome.

RGPL, RGP and BL could stimulate splenic lymphocyte proliferation in synergistic stimulation with PHA/LPS in vitro (Fig. 3B and C). Among them, stimulation of RGPL on splenic lymphocyte was the most significant under a concentration from 25 $\mu g\,mL^{-1}$ to 400 $\mu g\,mL^{-1}$. This finding indicated that stimulation on splenic lymphocyte proliferation of RGP was significantly improved after encapsulation with liposome in a synergistic manner.

A wealth of studies have shown that the therapeutic activity of drugs has been enhanced through encapsulation with liposomes (Krauze et al., 2007; Tardi, Dos Santos, et al., 2009; Tardi, Johnstone, et al., 2009; Yamashita et al., 2007). Drugs should be able to reach their target site at the right time, in the right concentration and at the right rate. At the present time, liposomes are the premier particulate carrier system under investigation. Liposomes are biocompatible, non-toxic, can be formulated in small size, have long circulating times and enhanced permeability and retention, thereby favorably altering drug bio-distribution (Avnir et al., 2008; Gabizon, Shmeeda, & Barenholz, 2003). Liposome-based formulations may also reduce the toxicity of drugs. In many cases, delivering the drug via an optimized liposomal formulation improves the therapeutic index of the drug. Furthermore, our previous studies have also found that the pharmacological activities of some Chinese herbal medicinal ingredients were enhanced after being encapsulated with liposomes (Fan et al., 2012; Gao et al., 2012; Yuan et al., 2012; Yuan et al., 2013; Zhao et al., 2012). Thus, better immunological enhancement of RGPL in vitro provides the theoretical basis for further experiments in vivo.

4. Conclusions

RSM is an effective statistical technique for optimizing complex processes. Based on the single-factor experiments, RSM was employed to estimate and optimize four experimental variables: the soybean phosphatide to cholesterol ratio (w/w), chloroform to methanol ratio (v/v), soybean phosphatide to tween 80 ratio (w/w) and temperature (°C) for the EE in the preparation process of RGPL. All of the independent variables and the quadratic of all of the independent variables had highly significant effects on the response values ($P < 0.02$). The optimal preparation conditions for the RGPL were as follows: a soybean phosphatide to cholesterol ratio of 8:1, a chloroform to methanol ratio of 3:5, a soybean phosphatide to tween 80 ratio of 10:1 and a temperature (°C) of 66 °C. Under these conditions, the experimental EE was 72.753 ± 0.318%, which was close to the predicted value (Table 4).

Liposomes are novel drug delivery systems and promising adjuvants. In the present study, a particular emphasis had been placed on the immunological enhancement advantages of the use of liposomes. From the results of RGPL on splenic lymphocyte proliferation in vitro, it could be concluded that stimulation on splenic lymphocyte proliferation of RGP was significantly improved after encapsulation with liposomes. Taken together, these findings indicated that the immunological enhancement of RGP was significantly enhanced after encapsulation with liposome, which provides the theoretical basis for further experiments in vivo.

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