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Optimization on conditions of *Lycium barbarum* polysaccharides liposome by RSM and its effects on the peritoneal macrophages function

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ABSTRACT

The purpose of this study was to optimize the preparation conditions of *Lycium barbarum* polysaccharides liposome (LBPL) by response surface methodology (RSM) and to investigate the effect of LBPL activating function of peritoneal macrophages. LBPL was prepared using the reverse-phase evaporation method. The optimal preparation conditions of LBPL by RSM were as follows: the ratio of lipid to drug (w/w) of 25:1, the ultrasound time of 14 min and the ratio of soybean phospholipids to cholesterol (w/w) of 2.4:1. Under these conditions, the experimental encapsulation efficiency of LBPL was 86.37 ± 0.63%, which was close to the predicted value. These indicated that LBPL with high entrapping efficiency and small particle size could be prepared by the reverse-phase evaporation method, which is applied easily. Furthermore, macrophages are the key players in the innate immune system. LBPL could effectively enhance peritoneal macrophages phagocytosis and resulted in inducing NO (nitric oxide) production in mouse peritoneal macrophages.

1. Introduction

Lycium barbarum (Solanaceae), which is popularly known as wolfberry, also named Goji, is an important Chinese herbal medicine and tonic (Bucheli et al., 2011; Lin, Lin, Chen, Ho, & Yeh, 2011b). LBP fractions generally consist of 6 monosaccharide (galactose, glucose, rhamnose, arabinose, mannose, and xylose) and 18 amino acids (Huang, Tan, Shen, & Lu, 1998; Gan, Zhang, Liu, & Xu, 2003). The water-soluble glycoconjugates have a molecular weight ranging from 24 to 241 kDa (Amagase, Sun, & Borek, 2009). LBP is used widely in Chinese herbal medicine for its immunomodulatory and antitumor effects such as enhancing the immune system (Gan et al., 2003; Wang et al., 2010), protecting the liver from damage (Wu et al., 2010), exhibiting anti-cancer (Gan, Hua Zhang, Liang Yang, & Xu, 2004). Considering crude extract and large molecules in the material, such as LBP, bioavailability is the issue for the in vivo effect (Amagase & Farnsworth, 2011). Thus, further studies are required to improve the bioavailability of LBP in clinical applications.

Liposomes present an effective drug delivery system (DDS): There are already more than 10 FDA-approved liposomal drugs and many more preparations are in clinical trials (Chang & Yeh, 2012; Wagner, Dullaart, Bock, & Zweck, 2006). Liposomal drugs are widely used in the pharmaceutical field since it has many advantages including the affinity to biological membrane and the ability to improve the unfavorable physicochemical properties of drugs (Li et al., 2009; Han, Shin, & Ha, 2012; Wang, Langer, & Farokhzad, 2012). In addition, liposomes have been studied as vaccine adjuvants, as they can function both as delivery systems and constitute a scaffold for the incorporation of different types of immunopotentiators (Henriksen-Lacey, Korsholm, Andersen, Perrie, & Christensen, 2011; Nordly, Madsen, Nielsen, & Foged, 2009).

Response surface methodology (RSM) is a collection of empirical and mathematical techniques used to evaluate the interactions between multiple parameters and optimize processes (Zhong, Lin,

Abbreviations: LBPL, *Lycium barbarum* polysaccharides liposome; LBP, *Lycium barbarum* polysaccharides; RSM, response surface methodology; LPS, lipopolysaccharide; PBS, phosphate buffered saline; EE, entrapment efficiency; BBD, Box–Behnken design; BL, blank liposome; NO, nitric oxide; DMEM, Dulbecco's modified Eagle's medium.

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Wang, & Zhou, 2012). This procedure reduces the number of experimental trials and overall cost (Myers & Montgomery, 2002).

Macrophages are a key player in the innate immune system and are versatile cells that play many roles. Activated macrophages play pivotal roles in inflammatory diseases via excess production of inflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2), as well as pro-inflammatory cytokines, to promote inflammatory responses (Vane et al., 1994; Marks-Konczalik, Chu, & Moss, 1998). Nitric oxide (NO) is a potent molecule involved in the non-specific cytotoxic effects mediated by macrophages against microorganisms. NO acts as an intracellular messenger and regulates cellular functions such as inflammation and the elimination of pathogens (Li, Xue, Geng, & Chen, 2012). In the present studies, production of NO was investigated to indicate the mechanism and functional consequences of LBPL induced macrophage activation.

To the best of our knowledge, there were no reports available in the literature regarding the optimization of the preparation conditions of LBPL by response surface methodology (RSM). In this work, the variables (the ratio of soybean phospholipids to drug (w/w), ultrasound time of liposome (min), the ratio of soybean phospholipids to cholesterol (w/w)) of preparing LBPL were firstly investigated and optimized using a BBD (3 factors and 3 levels). Enhancing peritoneal macrophages phagocytosis and inducing NO (Nitric oxide) production in mouse peritoneal macrophages of LBPL were investigated by various in vitro assays.

2. Materials and methods

2.1. Materials

Lycium barbarum polysaccharides (98% UV, No. CY131207), 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. Tween-80 (Sigma, No. 9005-65-6) was manufactured from Beijing Soledad Science and Technology Co., Ltd. Sephadex G-50 (No. 9048-71-9) was purchased from Hefei Hiromi Biological Technology Co., Ltd. Triton X-100 (No. 9002931) was obtained from Shanghai Yuanye Biotechnology Co., Ltd. RPMI-1640 (GIBCO) supplemented with penicillin 100 IU/ml, streptomycin 100 IU/ml. Lipopolysaccharide (LPS), (Sigma, No. L2880), as a B-cell mitogen, was dissolved into 0.05 mg/ml with RPMI-1640. LPS solution was filtered through a 0.22 μm syringe filter and was stored at −20 °C. Vybrant™ Phagocytosis Assay Kit (V-6694) was manufactured from Molecular Probes, Inc. DMEM (No. 1001648631) was obtained from Sigma–Aldrich for cell culture and Nitric Oxide Assay Kit (No. S0021) was manufactured from Beyotime Institute of Biotechnology.

2.2. Preparation and quality identification methods of BLP liposome

2.2.1. The method of preparing LBPL

LBPL was prepared by employing the reverse-phase evaporation method. In brief, the certain amount of soybean phospholipid, cholesterol and Tween-80 were dissolved in a solution with chloroform (4 ml) and ether (8 ml) by ultrasonic method to form an oil phase. Then, the aqueous phase was made by LBP dissolving into 4 ml phosphate buffer solution (PBS, pH 7.3). The mixture was homogenized using an ultrasonic cleaner in ice water, while aqueous phase by drops into the oil phase to form a stabile W/O emulsion. Organic solvent was later removed using a rotary vacuum evaporation under lipid transition temperature to form a colloid.

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

Factors	Code	Levels and range		
		−1	0	1
(A) ratio of soybean phospholipids to drug (w/w)	X_1	15	20	25
(B) the ultrasound time (min)	X_2	10	15	20
(C) ratio of soybean phospholipids to cholesterol (w/w)	X_3	1	2.5	4

And then PBS was added to hydrate for an additional 10 min. Next, the resulting mixture was homogenized using an ultrasonic cell disintegrator (JY92-II DN, Xinzhi Bio-technology and Science Inc.) for 10 min to form the small single-chamber liposomes. Finally, the liposome was filtered using 0.45 μm and 0.22 μm millipore membrane successively.

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

A modified mini-column centrifugation method was used to determine the encapsulation efficiency of LBPL (Fry, White, & Goldman, 1978). Briefly, Sephadex G-50 solution was prepared in 0.9% NaCl solution at last 5 h for complete swelling. Before Whatman filter pad was inserted in 2 ml syringe, removed the piston of the syringe. Then, the swollen Sephadex was added into it carefully to avoid air entrapment in the column. The excessive amount of water was removed by centrifuging the minicolumn at 1500 rpm for 5 min. 400 μl liposomes suspension was slowly added on prepared column, then centrifuged at 1500 rpm for 5 min. The same procedure was repeated by adding 400 μl saline to elute the remaining free drug (Xiong et al., 2009). The eluted liposomes were ruptured by using a 10% Triton X-100. The encapsulated drug was free and using the vitriol–phenol method to assay the content of encapsulated drug. The same method was applied to analyze the free drug and the total amount of liposomes. The formula to calculate the liposome encapsulation efficiency was $EE\% = Ce/Ct \times 100\%$, "*Ce*" is the amount of encapsulated drug and "*Ct*" is the total content of drug. The formula to calculate the drug-loading rate of liposome was $W\% = WE/WP \times 100\%$, WE is the amount of encapsulated drug, and WP is the total content of soybean phospholipid and cholesterol.

2.3. One-factor-at-a-time experiment of LBPL preparation

LBPL was prepared using the method as previously described in Section 2.2.1. The influence of four single factors (ratio of soybean phospholipids to drug, ultrasound time of liposome, rotary evaporation temperature, the ratio of soybean phospholipids to cholesterol) on the EE of LBPL were observed.

2.4. Optimization of LBPL preparative condition by RSM

On the basis of the single factor experiment, three independent variables at three levels (3^3) were adopted for RSM (Ye et al., 2012). These three independent variables were the ratio of soybean phospholipids to drug (w/w), ultrasound time of liposome (min) and ratio of soybean phospholipids to cholesterol (w/w), which were labeled as X_1, X_2 and X_3. Each variable was coded at three levels: −1, 0 and +1. The parameters and their levels were presented in Table 1, and the dependent variable *Y* was the entrapment efficiency (EE).

A three level-three variable BBD was adopted in this study, and 17 experiments were required to establish optimum conditions for the preparation of LBPL. The experiments were carried out in a

randomized order to minimize the influences of unexplained variability in the observed values, which might be caused by extraneous factor (Lin, Guo, Liu, & Afr, 2011a). A full quadratic equation for this model is shown as:

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} A_{ij} X_i X_j \quad (1)$$

where Y is the response variable, A_0 is a constant, A_i, A_{ii} and A_{ij} are coefficients for intercept, linear, quadratic and interactive terms, respectively, while X_i and X_j are independent variables ($i \neq j$). The coefficient of correlation (R^2) and analysis of variance (ANOVA) were used to evaluate suitability of the model. The models were compared based on the coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}) and predicted coefficient of determination (R^2_{pred}). The coefficient of determination (R^2) is defined as the regression of sum of squares proportion to the total sum of squares which illustrates the adequacy of a model. R^2 ranges from 0 to 1. R^2 values closer to 1, means the model is more accurate. The high adjusted and predicted coefficient of determination also illustrate whether the model adequately fits the data (Badwaik, Prasad, & Deka, 2012). After selecting the most accurate model, the analysis of variance (ANOVA) was used to investigate the statistical significance of the regression coefficients by conducting the Fisher's F-test at 95% confidence level.

2.5. Characterization of LBPL

The ultramicro morphous of liposomes were observed under transmission electron microscope (Model H-7650, Hitachi, High-Technologies Co., Ltd.). Briefly, LBPL was diluted with PBS, then added on copper screen and wrapped with carbon.

2.6. Mouse peritoneal macrophage preparation

Peritoneal macrophage cells were harvested three days after intraperitoneal injection of 1 ml of 6% starch-broth solution, and isolated using 5 ml PBS. Peritoneal cells were harvested, washed and resuspended in RPMI-1640 (2.5×10^6 cells/ml) with 10% fetal bovine serum. Cells were seeded in 6 well plastic plates (3 ml per well) and incubated at 37 °C in a 5% CO_2 humidified incubator overnight. The following day, removed non-adherent cells and washed gently twice times with RPMI 1640 medium. LBPL, LBP and BL were diluted with RPMI-1640 medium (without 10% FBS) at series of concentrations (250 μg/ml, 125 μg/ml, 62.5 μg/ml). In addition, negative control (commensurable RPMI-1640 medium) and positive control (10 μg/ml LPS) were set.

2.7. Phagocytic activity of peritoneal macrophages assay

After being treated with or without drug for 44 h, the peritoneal macrophages were harvested for phagocytosis activity analysis using Vybrant™ Phagocytosis Assay Kit. Briefly, harvested adherent cells using trypsin digestion method and removed to 1.5 ml EP tube. Then, cells were washed and added 100 μl FITC-labeled *E. coli*. After incubating in the dark at 37 °C for 2 h, excessive *E. coli* particles were removed with PBS. The trypan blue staining was to anchor cells, and the phagocytic activity was assessed via flow cytometry (BD FACSCalibur, Biosciences, Bedford, MA, USA). The phagocytosis response to the effector can then be expressed as follows:

$$\text{efficiency ratio (\%)} = \frac{\text{net experimental reading}}{\text{net positive reading}} \times 100\%.$$

2.8. Measurement of NO generation

Peritoneal macrophages were obtained as described as Section 2.6. The difference lies in the cells were cultured with DMEM medium (without phenol red) in 96 well plastic plates (100 μl per well). LBPL, LBP and BL were diluted with DMEM medium (without 10% FBS) at series of concentrations (125 μg/ml, 62.5 μg/ml, 31.25 μg/ml). After being treated with or without drug for 24 h, NO production in cell culture supernatants was measured using the Griess reagent (Beyotime, S0021) according to the manufacturer's instructions.

2.9. Statistical analysis

Data were analyzed by one-way ANOVA followed by Design-Expert 8.6.0 software where appropriate. Results are presented as means ± SE, and $p < 0.05$ was regarded as statistically significant. The fit quality of the polynomial model equation was expressed by R^2. The regression coefficients were used for statistical calculation to generate dimensional and contour maps from the regression models. All statistical analyses were performed with IBM SPSS Statistics 20.0 for Windows.

3. Results and discussion

3.1. Influence of four single factors on the encapsulation efficiency of LBPL

3.1.1. Influence of the ratio of soybean phospholipids to drug on the encapsulation efficiency of LBPL

The ratio of soybean phospholipids to drug was changed to 5:1, 10:1, 15:1, 20:1 and 25:1, and the other preparation conditions of LBPL were controlled at the same level. As the results shown in Fig. 1A, it is better that the ratio of the soybean phospholipids to drug was chosen at 20:1 in later experiments.

3.1.2. Influence of the ultrasound time of liposome on the encapsulation efficiency of LBPL

The ultrasound time was changed to 5 min, 10 min, 15 min, 20 min and 25 min, and the other preparation conditions of LBPL were controlled at the same level. According to the results shown in Fig. 1B, it is better that the ultrasound time was chosen at 15 min in later experiments.

3.1.3. Influence of the rotary evaporation temperature on the encapsulation efficiency of LBPL

The rotary evaporation temperature was changed to 25 °C, 35 °C, 45 °C, 55 °C and 65 °C, the other preparation conditions of LBPL were controlled at the same level. As shown in Fig. 1C, it is better that the rotary evaporation temperature was chosen at 45 °C in later experiments.

3.1.4. Influence of the ratio of soybean phospholipids to cholesterol on the encapsulation efficiency of LBPL

The ratio of soybean phospholipids to cholesterol was changed to 1:1, 2:1, 4:1, 6:1 and 10:1, and the other preparation conditions of LBPL were controlled at the same level. According to the results shown in Fig. 1D, it is better that the ratio of soybean phospholipids to cholesterol was chosen at 2:1 in later experiments.

3.2. Statistical analysis and the model fitting of the optimization of the preparation of LBPL

Response surface methodology is a set of mathematical and statistical techniques that are useful for modeling and predicting the response of interest affected by a number of input variables with the aim of optimizing this response (Montgomery, 2001). RSM also specifies the relationships among one or more measured responses and the essential controllable input factors. The influences of the ratio of soybean phospholipids to drug (w/w), ultrasound time

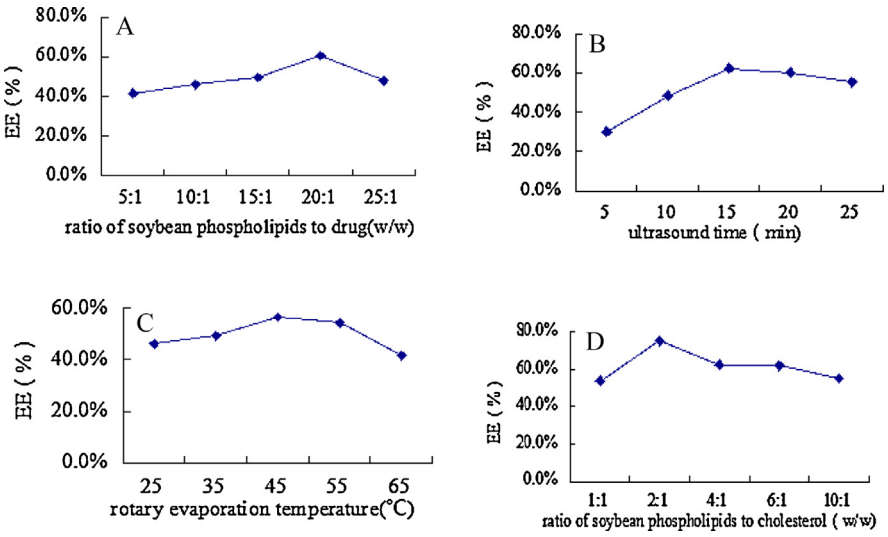

Fig. 1. The influence of different single factors on the EE (%) of LBPL.

of liposome (min) and ratio of soybean phospholipids to cholesterol (w/w) on the EE of LBPL were evaluated using Box–Behnken design (Table 2).The EE of LBPL ranged from 47.98% to 85.27%, and reached maximum with the ratio of soybean phospholipids to drug at 25:1, 14 min ultrasound time, and ratio of soybean phospholipids to cholesterol at 2.4:1. By applying multiple regression analysis and the data shown in Table 2 the predicted response Y for EE of LBPL could be obtained by the following second-order polynomial equation:

$$Y = +84.03 + 8.20X_1 - 6.88X_2 - 2.47X_3 + 4.72X_1X_2 + 1.16X_1X_3 + 6.44X_2X_3 - 4.76X_1^2 - 11.38X_2^2 - 10.54X_3^2 \quad (2)$$

The regression model was tested by ANOVA, as shown in Table 3. Lack of fit can be used to judge the validity of the model and the p-value was used to check the significance of the model (Stalikas, Fiamegos, Sakkas, Albanis, & Chromatogr, 2009). It can be seen that the lack of fit value of the mathematical model of 0.184 was not significant relative to the pure error. Based on the analysis of validity for the variables, it can be seen that the independent variables (X_1, X_2 and X_3), the interaction terms ($X_1 \times X_2$, $X_2 \times X_3$), and all two quadratic terms (X_1^2, X_2^2 and X_3^2) significantly ($p < 0.001$) affected the EE of LBPL. The statistical analysis indicated the proposed model was highly significant, possessing a very low p-value ($p < 0.0001$) and a very high F value ($F = 250.92$). The coefficient (R^2) was 0.9969, which was defined to explain the fitness of the regression models (Nath & Chattopadhyay, 2007). The bigger the value of R^2, the better relevant was the dependent variables in the model. The "Pred R-squared" of 0.9655 was in reasonable agreement with the "Adj R-squared" of 0.9929. In this study, it indicated a high degree correlation between the observed and the predicted values.

3.3. Optimization of LBPL preparation conditions

To show the type of interactions between the variables and the relationship between responses and experiment levels of each variable, the 3D response surface and 2D contour plots are profiled in Fig. 2. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not (Ye et al., 2012).

Fig. 2A depicts 3D response surface and 2D contour plots of the effects of the two variables, namely, the interaction effect of the

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

No.	Levels of independent factors			Response: EE (%)	
	X_1 (w/w)	X_2 (min)	X_3 (w/w)	Practical acquired EE	Predicted acquired EE
1	15:1	10	2.5:1	70.26	71.29
2	25:1	15	4:1	75.36	75.62
3	20:1	15	2.5:1	84.36	84.03
4	20:1	20	4:1	58.42	59.19
5	20:1	10	4:1	60.23	60.08
6	20:1	15	2.5:1	85.27	84.03
7	20:1	15	2.5:1	83.65	84.03
8	25:1	10	2.5:1	78.36	78.25
9	15:1	20	2.5:1	47.98	48.09
10	15:1	15	4:1	57.77	56.89
11	20:1	20	1:1	51.11	51.26
12	20:1	15	2.5:1	83.59	84.03
13	20:1	10	1:1	78.67	77.90
14	25:1	20	2.5:1	74.96	73.93
15	20:1	15	2.5:1	83.29	84.03
16	25:1	15	1:1	77.36	78.24
17	15:1	15	1:1	64.41	64.15

Table 3
ANOVA for response surface quadratic model.

Source	Sum of squares	df	Mean square	*F* value	Prob > *F*
Model	2447.23	9	271.91	250.92	<0.0001 Significant
A	538.25	1	538.25	496.7	<0.0001
B	378.81	1	378.81	349.57	<0.0001
C	48.86	1	48.86	45.09	0.0003
AB	89.11	1	89.11	82.23	<0.0001
AC	5.38	1	5.38	4.97	0.0611
BC	165.77	1	165.77	152.97	<0.0001
A^2	95.49	1	95.49	88.12	<0.0001
B^2	545.26	1	545.26	503.17	<0.0001
C^2	468.18	1	468.18	432.04	<0.0001
Residual	7.59	7	1.08		
Lack of fit	5.05	3	1.68	2.66	0.184 Not significant
Pure error	2.53	4	0.63		
Cor total	2454.81	16			

$R^2 = 0.9969$, $R_{Adj}{}^2 = 0.9929$, $R_{Pred}{}^2 = 0.9655$.

ratio of soybean phospholipids to drug (X_1) and the ultrasound time (X_2) on the EE of LBPL. The EE initially increased when there was an increase in the ratio of soybean phospholipids to drug and the ultrasound time. However, EE decreased after (X_1 = 25:1) and (X_2 = 14).

Fig. 2B represents the effects of the ratio of soybean phospholipids to drug (X_1) and ratio of soybean phospholipids to cholesterol (X_3) on the EE of LBPL, while the ultrasound time (X_2) was fixed. The ratio of soybean phospholipids to drug gave a slight increase in EE of LBPL whereas ratio of soybean phospholipids to cholesterol gave an exponential increase to EE within the 1:1 and 2.4:1. Then, EE gradually became smaller between 2.4:1 and 4:1.

The interaction effects of the ultrasound time and ratio of soybean phospholipids to cholesterol (X_2X_3) are shown in Fig. 2C, which indicated there was the strong interaction between the two factors. Both the ultrasound time and ratio of soybean phospholipids to cholesterol exerted a quadratic effect on the EE of LBPL. EE reached a peak when the ratio of soybean phospholipids to cholesterol was added to 2.4:1 and the ultrasound time was 14 min.

3.4. Characterization of liposome

The transmission electron microscope of LBPL was shown in Fig. 3. On the transmission electron micrograph, the particles showed nearly spherical shape with uniform size. In the preparation of liposomes we modified, Tween-80 was added as the surfactant and liposomes were actively loaded with LBP on aqueous phase by drops into the oil phase to form a stabile W/O emulsion. Given these adjustments, it was clear that liposomes were the small particle size and uniform distribution.

3.5. Verification of the predictive mode

To validate the adequacy of the model equation, three confirmation experiments were conducted under the optimized conditions (the ratio of lipid to drug (w/w) of 25:1, the ultrasound time of 14 min and the ratio of soybean phospholipids to cholesterol (w/w) of 2.4:1), and then responses were measured. A mean EE value of 86.37 ± 0.63% (n = 3) was obtained. The maximum predicted and

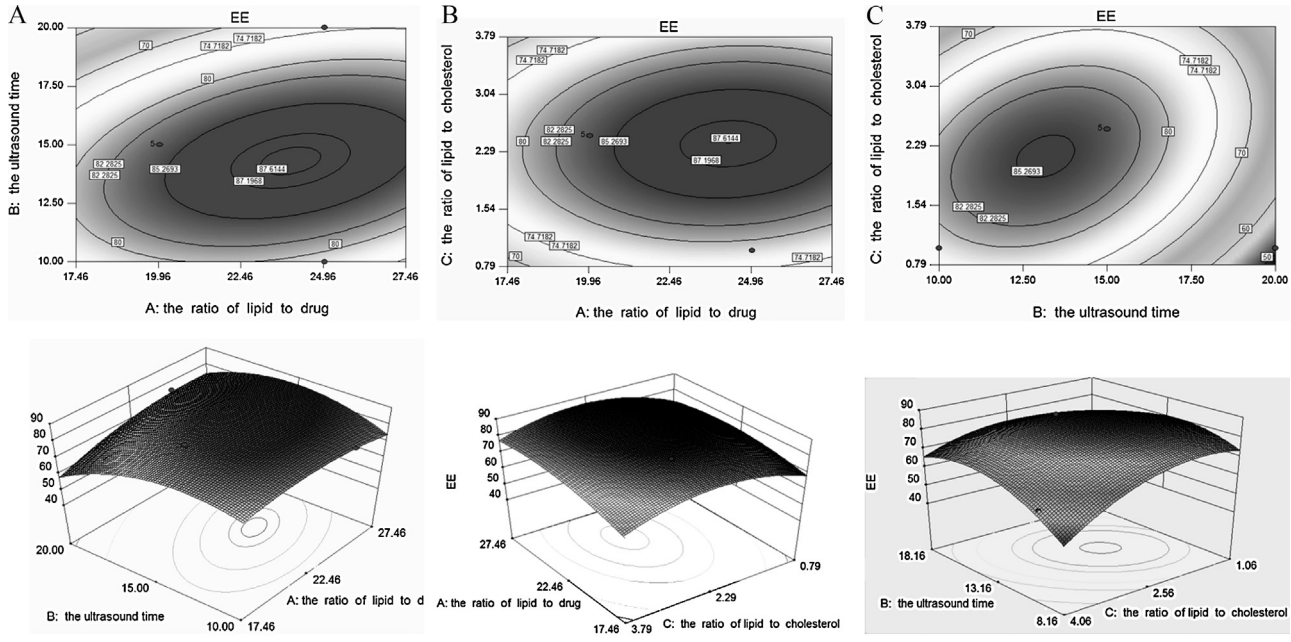

Fig. 2. (A) 3D response surface and 2D contour plots for the effects of the ratio of soybean phospholipids to drug and the ultrasound time. (B) 3D response surface and 2D contour plots for the effects of the ratio of soybean phospholipids to drug and ratio of soybean phospholipids to cholesterol. (C) 3D response surface and 2D contour plots for the effects of the ultrasound time and ratio of soybean phospholipids to cholesterol.

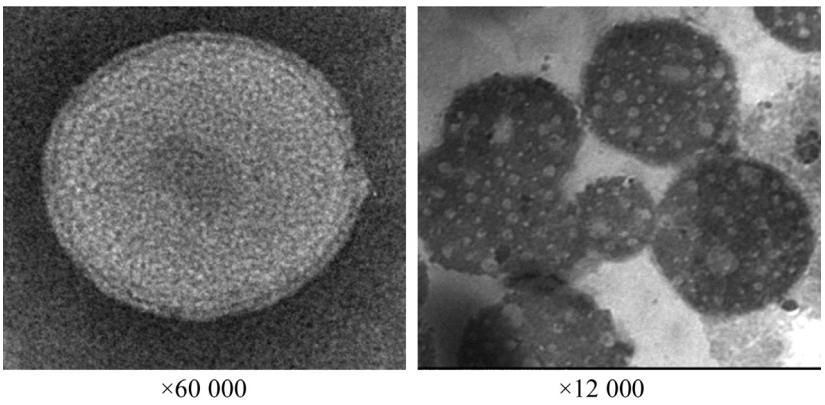

Fig. 3. Transmission electron micrograph of LBPL without staining.

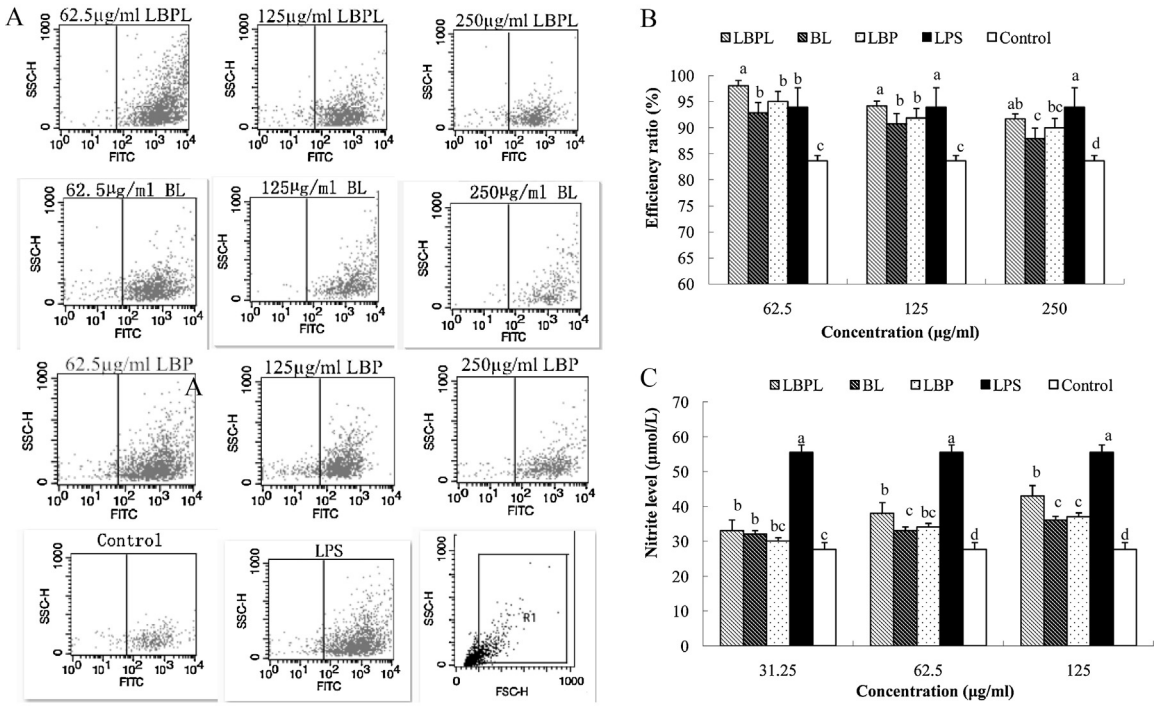

Fig. 4. (A) Dot-plot of FSC-SSC and FL1-histogram of mouse peritoneal macrophages incubated with fluorescent *E. coli* k-12 Bioparticle. (B) The efficiency ratio of the phagocytosis response on each group. [a–d] Bars in the figure without the same superscripts differ significantly ($p < 0.05$). (C) The nitric oxide of the peritoneal macrophages on each group (μM/ml). [a–d] Bars in the figure without the same superscripts differ significantly ($p < 0.05$).

experimental value of EE was given in Table 4. The results indicated that the RSM approach was effective for optimizing the conditions for the preparation of LBPL.

3.6. LBPL could enhance the phagocytic activity of the peritoneal macrophages

Macrophages are major inflammatory and immune effector cells closely related to phagocytic cells that cooperate during the onset, progression and resolution of inflammation (Soehnlein & Lindbom, 2010). Phagocytosis is an important mechanism for nourishment in unicellular organisms and for defense against infection in higher vertebrates. Macrophages can be activated by different stimuli, or combination of stimuli, and differentiate into cells exerting diverse functions (Yu et al., 2013). In Fig. 4A, the area of peritoneal macrophages was marked out by Dot-plot of all cells based on FITC and SSC-H. R1 represented the gate of macrophages. As Fig. 4A showed, the phagocytic cells of three LBPL groups were

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

	Ratio of soybean lipid to drug (w/w)	Ultrasound time (min)	Ratio of soybean lipid to cholesterol (w/w)	EE (%)
Optimum conditions	25:1	14.4	2.36:1	87.671
Modified conditions	25:1	14	2.4:1	86.37 ± 0.63

more intensive than other control groups. As percent of positive fluorescence cells in total cells, the efficiency ratio of the phagocytosis response expressed the flow cytometry data in Fig. 4B. The efficiency ratio of the positive fluorescence cells with LBPL stimulating was higher than other control groups ($p < 0.05$). Moreover, the low concentration group of LBPL had the highest efficiency ratio and the phagocytosis response. We also found that the concentration of 62.5 μg/ml LBPL group is stronger than LPS in enhancing the phagocytic activity of the peritoneal macrophages.

3.7. LBPL could promote the NO production of the peritoneal macrophages

During the past two decades, nitric oxide (NO) has been recognized as one of the most versatile players in the immune system. The upregulation of surface receptors synergistically activates the MAPKs (p38 and JNK) which in turn induce the NF-κB activation and its translocation to the nucleus, resulting in enhanced expression of NO production in mouse peritoneal macrophages(Chakraborty, Bhatt, & Sodhi, 2012). The results of recent research show that NO plays an important role in the process of activated macrophage killing the tumor cells. LPS have been reported to induce NO expression through TLR4/p38/NF-κB signaling pathway in macrophages (Chen & Wang, 1999; Chen, Chen, & Lin, 1999). As shown in Fig. 4C, the concentrations of 125 μg/ml, 62.5 μg/ml and 31.25 μg/ml LBPL groups were higher than those in other groups except for LPS group. Especially, 125 μg/ml of LBPL group could significantly promote the NO production of the peritoneal macrophages ($p < 0.05$). In our study, LBPL had obviously enhanced the NO secretion of the peritoneal macrophages.

4. Conclusions

In this study, we found that the preparation conditions of *Lycium barbarum* polysaccharides liposome (LBPL) was optimize by response surface methodology (RSM). Numerical optimization determined the optimum preparation conditions to be the ratio of lipid to drug (w/w) 25:1, the ultrasound time 14 min and the ratio of soybean phospholipids to cholesterol (w/w) 2.4:1. In addition, the experimental encapsulation efficiency of LBPL was 86.37 ± 0.63%. As reported previously, liposomes are novel drug delivery systems and promising adjuvants. In our work, the LBP was encapsulated in the liposome using the reverse-phase evaporation method to improve the effects of LBP. The results showed that the LBPL had enhanced the phagocytosis activation and secretion of nitric oxide (NO) of the mouse peritoneal macrophages in vitro. These findings proposed a well characterized novel LBPL formulation as drug delivery system, and its mechanism and application will be further studied.

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