Carbohydrate Polymers 117 (2015) 510–517

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Development of liposomal *Ganoderma lucidum* polysaccharide: Formulation optimization and evaluation of its immunological activity

Zhenguang Liu[a], Xia Ma[b], Bihua Deng[c], Yee Huang[a], Ruonan Bo[a], Zhenzhen Gao[a], Yun Yu[a], Yuanliang Hu[a], Jiaguo Liu[a], Yi Wu[a], Deyun Wang[a,*]

[a] Institute of Traditional Chinese Veterinary Medicine, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, PR China
[b] Henan University of Animal Husbandry & Economy, Zhengzhou 450011, PR China
[c] National Research Center of Veterinary Biologicals Engineering and Technology, Jiangsu Academy of Agricultural Science, Nanjing 210014, China

ARTICLE INFO

Article history:
Received 4 June 2014
Received in revised form
24 September 2014
Accepted 26 September 2014
Available online 15 October 2014

Keywords:
Ganoderma lucidum polysaccharides
liposome
Optimization
Response surface methodology
Lymphocyte proliferation

ABSTRACT

The aim of this study was to investigate the optimizing preparation conditions of *Ganoderma lucidum* polysaccharide liposome (GLPL) with response surface methodology (RSM) and the immunological enhancement activity of GLPL. The immunological enhancement activity of GLPL on splenocyte proliferation was measured. The optimum formulation of GLPL, in which the ratio of soybean phospholipid to cholesterol(w/w) of 11:1, the ratio of soybean phospholipid to tween-80 (w/w) of 10.5:1 and ultrasonic time(min) of 11, had higher entrapment efficiency (EE) of 71.43 ± 0.49% with spherical shape and uniform sizes. In addition, GLPL could significantly promote splenocyte proliferation singly or synergistically with PHA and LPS. That indicated that the immunological enhancement of Ganoderma lucidum polysaccharide (GLP) was significantly enhanced after encapsulation with the liposome.

1. Introduction

Ganoderma lucidum, an oriental fungus called "ShenNong's Materia Medica", has been widely used as a traditional Chinese herbal medicine in improving immunity and human health for centuries (Berovič et al., 2003; Zhou et al., 2007). Many clinical and experimental studies show that this fungus has powerful immunomodulating, anti-tumoractivity (Lin, 2005; Yue, Fung, Leung, & Lau, 2008) and so on. It has been reported that the water-solutble polysaccharides are the main biologically active ingredients in *Ganoderma lucidum*. The composition of *Ganoderma lucidum* polysaccharide (GLP) is composed of D-glucose, D-galactose, D-mannose, D-xylose, l-fucose, and l-rhamnose, with MW 3.7×10^4D, and with 5.35:2.67:1:1.19:0.38:0.37 in molar ratio (Bao, Wang, Dong, Fang, & Li, 2002).Research demonstrates these physiological and health effects, including strong antioxidant activities (Xu et al., 2009), immunomodulating activities (Lin et al., 2006) and anti-tumor activities (Paterson, 2006). GLP not only enhanced lymphocyte proliferation and humoral immunity, but also promote expression of IFN-γ gene in chickens, which showed that GLP was a potential immune adjuvant (Zhang et al., 2014). However, the GLP also have some disadvantageous characteristics such as non-focused action scope, dose-dependent and a brief biological half-life, which can limit its application in the future.

Liposomes have been studied extensively for development of an efficient drug delivery system. It is the most promising among the numerous particulate delivery systems. Liposomes are often composed of lipids that occur naturally in cell membranes, including soybean phospholipid and cholesterol, which are almost non-toxic and completely biodegradable and have excellent affinity on cell membrane (Watson, Endsley, & Huang, 2012). Additionally, not only hydrophobic drugs can be encapsulated in liposome, but also liposomes have low permeability to hydrophilic drugs (Allen & Cullis, 2013). The efficacy of many drugs can be improved by the encapsulation of liposomes. Plenty of researchers confirmed that after the Chinese herbal medicinal ingredients are made with liposomal formulations, their immunocompetent were significantly increased (Yu et al., 2013).

In order to optimise the preparation conditions for the preparation of GLPL, response surface methodology (RSM) has been used to select the optimum preparation conditions. In statistics, RSM has

Abbreviations: GLPL, Ganoderma lucidum polysaccharide liposome; GLP, *Ganoderma lucidum* polysaccharide; RSM, response surface methodology; PHA, phytohemagglutinin; LPS, lipopolysaccharide; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; EE, Entrapment efficiency; DMSO, dimethyl sulfoxide; BBD, Box-Behnken design; BL, blank liposome.

* Corresponding author. Tel.: +86 25 84395203; fax: +86 25 84398669.
E-mail address: dywang@njau.edu.cn (D. Wang).

http://dx.doi.org/10.1016/j.carbpol.2014.09.093
0144-8617/

successfully been applied in order to optimize the conditions in food and pharmaceutical research (Ghadiri et al., 2012; Yang, Chen, Zhao, Han, & Chen, 2012; Yolmeh, Najafi, Farhoosh, & Hosseini, 2014). By establishing a mathematical model, RSM can effectively optimise complex preparation conduction in statistical way so to reduce the number of experimental trials (Sinha, Chowdhury, Saha, & Datta, 2013; Xu et al., 2008).

Within this framework, we investigated the preparation of GLPL using the reverse-phase evaporation method and established the optimum conditions by RSM. In addition, in order to compare the immunological enhancement activity of GLPL with GLP in vitro, the splenocyte proliferation were measured. The goal of this study is to improve immunological enhancement activity of GLP by liposome delivery system.

2. Materials and methods

2.1. The preparation of GLPL

Preparation of GLPL was based upon the reverse-phase evaporation method. The required amount of soybean phospholipid (Shanghai Taiwei Pharmaceutical Co., Ltd), tween-80 (Sinopharm Chemical Reagent Co., Ltd.) and cholesterol (Tianjin Bodi Chemical Technology Co., Ltd.) was dissolved by ethylether in a dry round bottom flask. With the help of ultrasonic cleaner, the materials were dissolved completely. Then GLP (98%, No.CY140220, Shanxi Ciyuan Biotechnology Co. Ltd.) solution was added into the mixture. The resulting mixture was homogenized in ice water by using an ultrasonic cleaner to form a stabile W/O emulsion. The solution was evaporated and a colloid was formed using a vacuum. 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.) to form the uniform liposome. Ultimately, the solution was successively filtered using 0.8 μm, 0.45 μm and 0.22 μm millipore membrane.

2.1.1. Entrapment efficiency (EE) of GLPL array

A modified protamine method was used to assay the EE of GLPL according to reference (Gunter, Gunter, Jarkowski, & Rosier, 1982).

In brief, 0.1 mL of protamine (Sigma) solution (10 mg mL^{-1}) was added in 0.1 mL of GLPL, the mixture was placed for 3 min. Next, 3 mL of PBS was added. After adequate mixing, this suspension was centrifuged at 3500 rpm at room temperature for 30 min. All of the supernatant was taken from the centrifuge tube and 2 mL of the supernatant was used to assay the content of GLP using the vitriol–phenol method, and the content of GLP was referred to as the content of free drug. In addition, 0.1 mL of GLPL was added in the centrifuge tube was mixed with 0.1 mL of Triton X-100 (Shanghai Yuanye Biotechnology Co., Ltd.) and 3 mL of PBS. Subsequently, 2 mL of the solution was used to assay the content of GLP using the vitriol–phenol method (Yu, Zhang, & Zheng, 2003), and this solution was called the amount of drug in 0.1 mL of liposomes suspension.

The formula to calculate the liposome encapsulation efficiency was expressed as follows:

$$EE\% = (1 - Q_f/Q_t)100\%$$

where Q_f is the amount of free drug and Q_t is the amount of drug in 0.1 mL of liposomes suspension.

2.2. Optimization design and characterization of GLPL

2.2.1. RSM and experimental design

On the basis of the preliminary range of parameters by a single-factor experiment for the GLPL preparation, the three factors, ratio of soybean phospholipid to cholesterol (w/w) (A), ratio of soybean phospholipid to tween-80 (w/w) (B) and ultrasonic time/min (C), were found significantly affect the entrapment efficiency of GLPL. Therefore, three level-three variable BBD was applied in this study, and series of 17 experiments were carried out to optimize conditions for the preparation of GLPL. Three factors and their levels were performed in Table 1.

In order to predict the optimized conditions, experimental data were analyzed using software Design Expert Version 8.06 and explained using the following quadratic equation:

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

Where Y is the predicted response variable associated with each three level combination, A_0 is a constant, and A_i, A_{ii} and A_{ij} are coefficients which were estimated by the model. X_i and X_j are the levels of the independent variables which represent the linear, quadratic and cross-product effects of the X_1, X_2 and X_3 factors on the response, respectively. The model evaluated the effects of each independent variable on the response. Analysis of the experimental design and calculation of predicted data were calculated using Design-Expert Software to estimate the response of the independent variables. (Wang, Chen, Jia, Tang, & Ma, 2012)

2.2.2. Characterization of liposomes

The morphologies of liposomes were observed using transmission electron microscope (Model H-7650, Hitachi, High-Technologies Co., Ltd.), samples were negatively stained with 1% phosphotungstic. The particle size of GLPL was measured by Hydro2000Mu laser particle size analyzer (Hydro2000Mu, MAL 1009117, Malvern Instruments Ltd.). The measurements were conducted at 25 °C and each experiment was repeated three times.

2.3. Splenocyte proliferation assay in vitro

Spleen was collected from non-immunized ICR mice under aseptic conditions, and kept in phosphate-buffered saline solution. The organ was crushed and passed through a steel mesh (200 mesh) to obtain a homogeneous cell suspension. After the red blood cells were removed by adding 0.83% NH_4Cl in 0.01 M Tris–HCl (pH 7.2) to cell suspension, the splenocytes were washed twice. Next, the pelleted cells were re-suspended in RPMI-1640 (RPMI-1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU mL^{-1} penicillin, 100 μg100 IU mL^{-1} streptomycin and 10% heat inactivated FBS) and diluted to 2.5×10^6 mL^{-1} with RPMI-1640 in FBS. The cell suspension was divided into two parts: one part was added, with PHA or LPS and seeded into a 96-well microtiter plate with 100 μL per well, and the other part was added as cell control group.Then, GLP, GLPL 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 (10 μg mL^{-1} of final concentration) or LPS (5 μg mL^{-1} of final concentration), respectively, at 100 μL per well, four wells for each concentration. The plates were incubated at 37 °C in humidified incubator with 5% CO_2 (Thermo) for 48 h. All the tests were carried out in triplicate and the cell proliferation was evaluated using MTT method. Briefly, 30 μL of MTT solution (5mg mL^{-1}) were added to each well 4 h before the end of incubation. After centrifugation of the plates (2000 rpm, 5 min), untransformed MTT was removed carefully by pipetting. Then 100 μL of DMSO (produced Zhengxing Institute of Chemical Engineering in Suzhou, China.) was added into each well to dissolve the formazan crystals. 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

Table 1
Uncoded and coded levels of the independent variables of the EE for the Box–Behnken.

Variable	Symbols uncodeed	Coded	Coded levels		
			−1	0	1
Soybean phosphatide to cholesterol ratio (w/w)	*X*1	A	8:1	10:1	12:1
Soybean phosphatide to tween 80 ratio (w/w)	*X*2	B	6:1	9:1	12:1
Ultrasonic time(min)	*X*3	C	5	10	15

assay reader (Model RT-6000, Leidu Co., Ltd. Shenzhen City) at a wave length of 570 nm (Huang et al., 2014).

2.4. Statistical analysis

Design Expert Version 8.06 was used to analyze the experimental data of the optimization of GLPL preparation and the second-order polynomial equation, ANOVA of the quadratic regression model, the optimal conditions were showed. By analyzing the response surface contour plots, the interaction among the corresponding function on the response and the different independent variables was analyzed. Data of the Splenocyte proliferation experiment were expressed as the mean ± standard Deviation (S.D.). Duncan and LSD's multiple range test were used to compare the parameter between groups. *P* values of less than 0.05 were considered statistically significant.

3. Result and discussion

3.1. Statistical analysis and model fitting

RSM as an effective means for optimization was used to optimize the preparation of GLPL. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes. It solves multivariable data which is obtained from properly designed experiments to solve multivariable equations simultaneously. A standard Box–Behnken design (BBD) was applied as design of experiment to identify the relationship between the response function and the process variables.

According to the single-factor experiment results, the coded factor levels and real values of three factors (ratio of soybean phospholipid to cholesterol (w/w), ratio of soybean phospholipid to tween-80 (w/w) and ultrasonic time(min) are adopted in this study (Table 1). There was a 17-run BBD with three factors and three levels for fitting a second-order response surface to optimize the preparation conditions (Table 2). The experimental sequence was randomized in order to minimize the effects of uncontrolled factors. It can be seen from Table 2 that there is considerable variation in EE depending upon preparation conditions. These results also showed that the EE of GLPL ranged from 37.53% to 71.04%. The highest EE value (71.04%) was found in conditions of $A = 10$, $B = 9$ and $C = 10$. The data obtained from the three level BBD matrix yielded the following regression equation (Eq. (1)), which was an empirical relationship between EE and the test variables.

By statistically processed and fitting, the multiple second-order equations were obtained as follows: The coded form:

$$Y = 69.84 + 6.17A + 4.14B + 1.08C + 1.98AB + 5.87AC + 3.54BC - 9.47A^2 - 5.31B^2 - 11.02C^2 \quad (2)$$

The ANOVA was carried out to determine the significance of the response surface quadratic model and significance of the regression coefficients were evaluated by their corresponding *F*-value and *P*-values in Table 3. And the *F*-test had a high model *F*-value (80.98) and a very low *P*-value ($P < 0.0001$). The smaller the *P*-values were, the bigger the significance of the corresponding coefficient was (Murthy, Swaminathan, Rakshit, & Kosugi, 2000), which implied the model was suitable for use. The Model *F*-value 80.98 implied that model was significant. The determination coefficients (R^2) and the multiple correlation coefficients (R) were used to confirm the quality of the model. In our study, the coefficient of determination (R^2) of the model was 0.9905 in Table 3, which indicated good agreement between the experimental and predicted values of EE. The *F*-value and *P*-value of "lack-of-fit" was 4.18 and 0.1004, respectively, which indicated that the "lack-of-fit" was insignificant relative to the pure error. It indicates that the model equation was adequate for predicting EE under any combination of values of the variables.

3.2. Optimization of preparation condition

The 3-D response surface and 2-D contour plots that are the graphical representations of regression equation obtained from the calculated response surface are indicated in Fig. 1. They showed three independent response surface plots and their respective contour plots, which provide a means of visualizing the relationship between the responses and experimental levels of each variable and the type of interactions between the two independent variables. The shapes of the contour plots (circular or elliptical) indicate whether the mutual interactions between the variables are significant or not. Circular contour plots indicate that the interactions between the corresponding variables are negligible, while elliptical contour plots indicate that the interactions between the corresponding variables are significant (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). In the project, three independent response surface plots and their respective contour plots were obtained using Design-Expert 8.06 as shown in Fig. 1.

In Fig. 1, the interactions between two independent variables and their optimum ranges can be obtained. It is clear that EE changes with minor alterations of the test variables (ratio of soybean phospholipid to cholesterol, ratio of soybean phospholipid to tween-80, and ultrasonic time). In addition, three interactions (ratio of soybean phospholipid to cholesterol and ratio of soybean phospholipid to tween-80, ratio of soybean phospholipid to cholesterol and ultrasonic time, ratio of soybean phospholipid to cholesterol and ultrasonic time) among the tested variables are significant ($P < 0.05$). Through analyzing these 3D response surface and their respective contour plots, it is convenient to understand the interactions between two independent variables and to locate their optimum ranges. In addition, the predicted EE was observed as 69.84% and found to lie in the following ranges of the examined variables: (ratio of soybean phospholipid to cholesterol being from 10.05:1 to 11.43:1, ratio of soybean phospholipid to tween-80 being from 9.10:1 to 11.49:1, and ultrasonic time being from 9.21 to 11.94 min).

The optimum conditions ($A = 10.93$, $B = 10.69$, and $C = 11.32$) for EE were estimated using the model equation by solving the regression equation and analyzing the response surface contour plots. Under the above conditions, predicted values were 72.58% from fitted equations, slightly higher than that obtained from plots analysis.

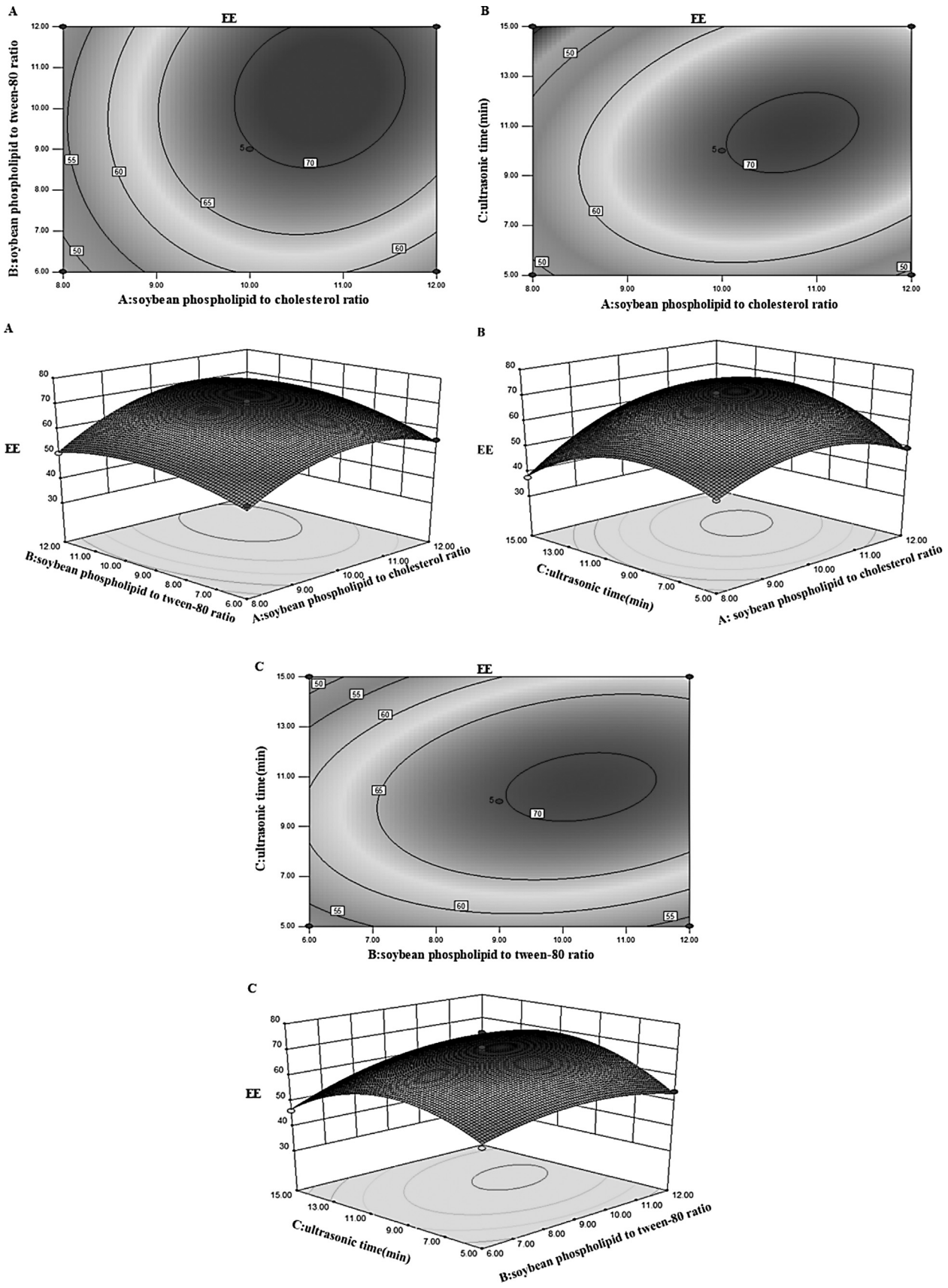

Fig. 1. 2-D contour plots and 3-D response surface plots showing effects of various parameters on EE.

Table 2
The BBD matrix and the experimental data for the responses.

No.	Levels of independent factors			Response EE (%)	
	A	B	C	Practical acquired EE	Predicted acquired EE
1	12:1	6:1	10	55.71	55.10
2	8:1	6:1	10	48.33	46.72
3	12:1	12:1	10	65.73	67.34
4	8:1	12:1	10	50.43	51.04
5	12:1	9:1	5	49.42	48.56
6	8:1	9:1	5	47.82	47.96
7	12:1	9:1	15	62.6	62.46
8	8:1	9:1	15	37.53	38.39
9	10:1	6:1	5	50.35	51.82
10	10:1	12:1	5	53.78	53.03
11	10:1	6:1	15	46.16	46.91
12	10:1	12:1	15	63.73	62.26
13	10:1	9:1	10	69.11	69.84
14	10:1	9:1	10	70.54	69.84
15	10:1	9:1	10	69.93	69.84
16	10:1	9:1	10	68.56	69.84
17	10:1	9:1	10	71.04	69.84

Table 3
Analysis of variance of the experimental results of the BBD.

Source	Sum of squares	df	Mean square	*F* value	*P*-value Prob > *F*
Model	1768.56	9	196.51	80.98	<0.0001 significant
A	304.43	1	304.43	125.46	<0.0001
B	137.12	1	137.12	56.51	0.0001
C	9.35	1	9.35	3.85	0.0904
A × B	15.68	1	15.68	6.46	0.0385
A × C	137.71	1	137.71	56.75	0.0001
B × C	49.98	1	49.98	20.6	0.0027
A × A	377.94	1	377.94	155.75	<0.0001
B × B	118.80	1	118.80	48.96	0.0002
C × C	511.26	1	511.26	210.69	<0.0001
Residual	16.99	7	2.43		
Lack of Fit	12.88	3	4.29	4.18	0.1004 not significant
Pure Error	4.11	4	1.03		
Cor Total	1785.548	16			

$R^2 = 0.9905$; $R^2_{Adj} = 0.9783$; $R^2_{Pred} = 0.881$.

The suitability of the model equation for predicting the optimum response values was tested using the recommended optimum conditions. Taking convenience into account, the optimum experimental parameters were determined as follows: $A = 11{:}1$; $B = 10.5{:}1$; and $C = 11$. Then EEs of each batch were determined. The mean experimental EE $71.43 \pm 0.49\%$ was close to the predicted value of 72.58% (Table 4), which showed that the response model was adequate for the preparation process.

Response surface methodology (RSM) has been commonly used for the optimization of formulations with drug in the development of formulation design (Gupta, Assmus, Beckert, & Price, 2001; Ko, Park, Park, Hwang, & Park, 2003). It is an effective and powerful statistical method for optimization on preparation of nanoparticles while reducing the number of experimental trials required. The quality of drug liposome is affected by encapsulation efficiency of drugs and the high encapsulation efficiency is the critical factor to decide the clinical curative effect of drugs (Xu, Zhang, & Wang, 2004). In the study, we selected encapsulation efficiency as the response and optimized the preparation condition of GLPL using RSM. The result showed high encapsulation efficiency ($71.43 \pm 0.49\%$) was prepared under the optimal conditions. Under optimal conditions, GLPL was prepared and its immunological activity was further investigated.

Table 4
Optimized values obtained by constraints applied on EE.

Variable and response	Optimum condition	Modified condition
A (w/w)	10.93:1	11:1
B (w/w)	10.69:1	10.5:1
C (min)	11.32	11
EE (%)	72.58	71.43 ± 0.49

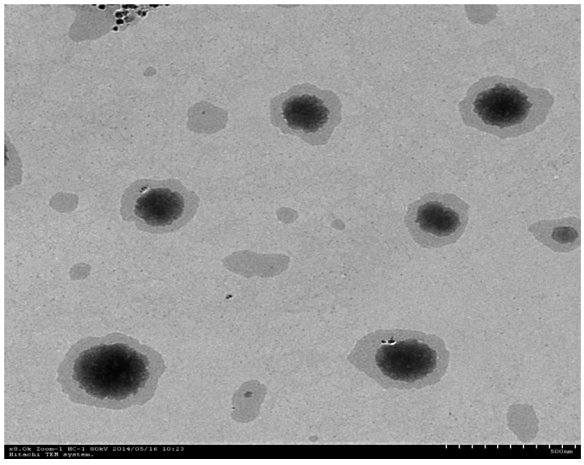

Fig. 2. Transmission electron micrograph of GLPL.

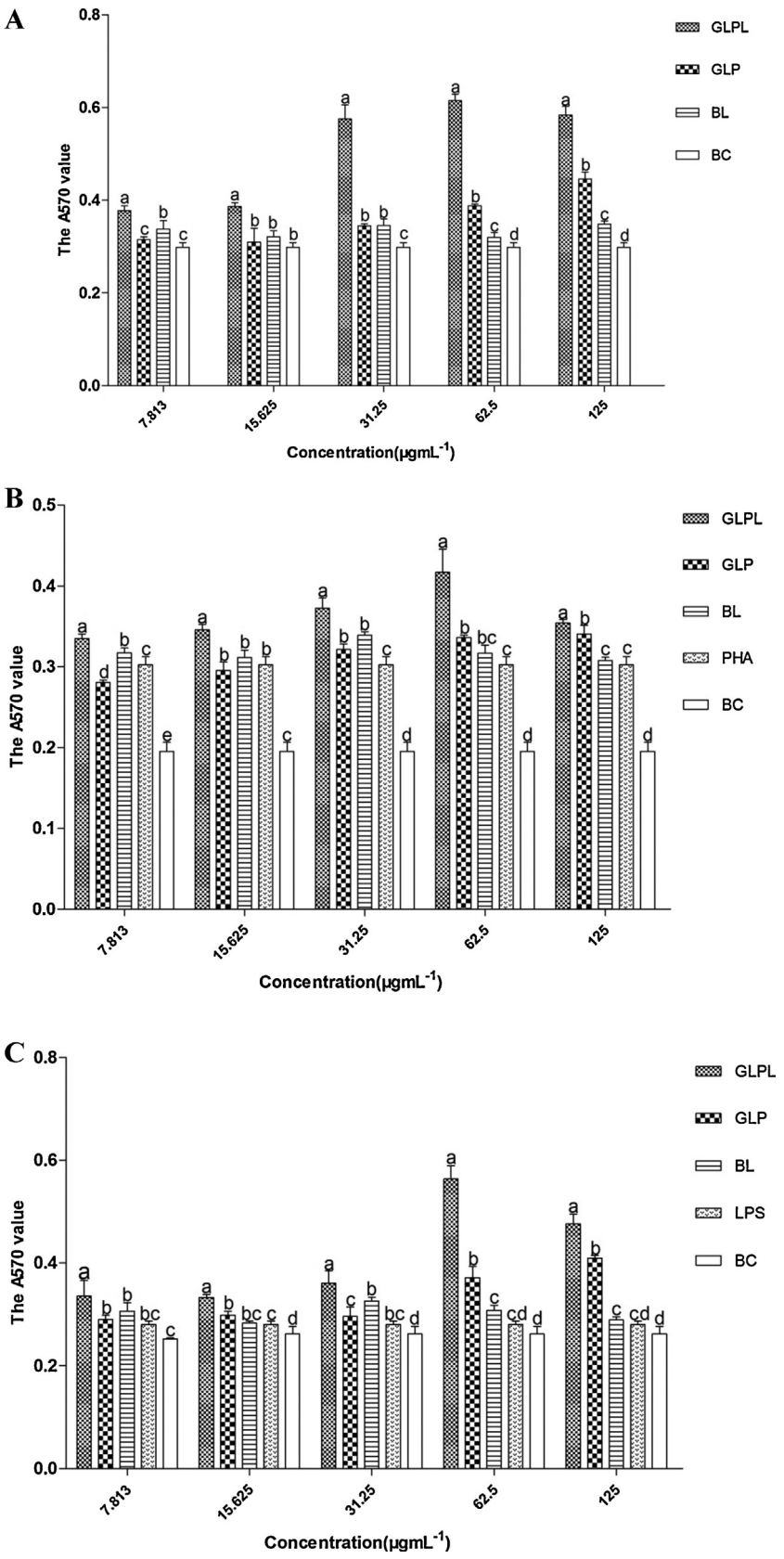

Fig. 3. (A) The change of splenic lymphocyte proliferation in single stimulation of drugs (A_{570}). $^{a-c}$Bars in the figure without the same superscipts differ significantly ($P < 0.05$). (B) The change of splenic lymphocyte proliferation in synergistical stimulation of drugs with PHA (A_{570}).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

3.3. Characterization of GLPL

Macroscopic appearance of the GLPL showed the homogeneous slight yellow–white and translucent suspension. By Transmission electron micrograph of GPSL, GLPL appeared nearly spherical shape and the monodisperse vesicles (Fig. 2). The particle diameters for GLPL were 164.3 ± 9.2 nm. Mean size and size distribution values are physicochemical parameters that have to be modulated as a function of the proposed application for a certain liposomal system (Grazia Calvagno et al., 2007). The well characterized novel GLPL formulation was manufactured and it was selected for further experimentation.

3.4. Splenocyte proliferation assay in vitro

The spleen plays an important role as main immune organ in preventing disease. The splenic lymphocytes proliferation was used as important indicator to study immune response and evaluate cellular immune function. After stimulated by antigenic properties, T and B lymphocytes could proliferate and differentiate, generate specific immune response, and produce lymphokine and antibody, they are considered as the most important immune effector cell (Minato et al., 2004). By determining the proliferation of splenic lymphocytes, the activity and action mechanism of drugs can be evaluated preliminary. To investigate the role of GLPL on immunological enhancement, the effect on splenic lymphocyte proliferation was examined.

The effect of spleen lymphocyte proliferation in single stimulation of drugs was shown in Fig. 3A. The A_{570} values of GLPL gradually became higher from 7.813 μg mL^{-1} to 62.5 μg mL^{-1} but slightly lowered after 62.5 μg mL^{-1}. The A_{570} values of GLPL were significantly higher compared with those of GLP and BL at the same concentration ($P < 0.05$) and the highest A_{570} value was at 62.5 μg mL^{-1}.

The effect of spleen lymphocyte proliferation in synergistical stimulation of drugs with PHA was shown in Fig. 3B. The A_{570} values of GLPL became higher from 7.813 μg mL^{-1} to 62.5 μg mL^{-1} but lowered after 62.5 μg mL^{-1} after synergistic stimulation with PHA. The A_{570} values of GLPL were significantly higher compared with those of GLP and BL under the same concentration ($P < 0.05$) and the highest A_{570} value was at 62.5 μg mL^{-1} after synergistic stimulation with PHA.

The effect of spleen lymphocyte proliferation in synergistical stimulation of drugs with LPS was shown in Fig. 3C. The A_{570} values of GLPL slightly became higher from 7.813 μg mL^{-1} to 62.5 μg mL^{-1} but lowered to 62.5 μg mL^{-1} after synergistic stimulation with LPS. The A_{570} values of GLPL were significantly higher compared with those of GLP and BL at the same concentration ($P < 0.05$) and the highest A_{570} value was at 62.5 μg mL^{-1}.

The results of this study showed that the A_{570} values of GLPL at five concentration groups were the highest not only in single stimulation but also in synergistical stimulation with PHA or LPS, and significantly higher than those in BL and GLP at most concentration groups. It demonstrated that both T and B cells were activated by GLPL and the immune enhancement activity of GLP was significantly improved with combination of GLP and liposome delivery system.

Liposomes, as the first closed bilayer phospholipid systems, were described in 1965 and soon were proposed as drug delivery systems. They have successfully been used to encapsulate toxic drugs and deliver them as a non-toxic and more effective therapeutic (Gruber et al., 1989; Konigsberg, Godtel, Kissel, & Richer, 1998). Liposomes have led to numerous clinical trials in such diverse areas as the delivery of anti-cancer, anti-fungal and antibiotic drugs, the delivery of gene medicines, and the delivery of anesthetics and anti-inflammatory drugs (Allen & Cullis, 2013). The ability of liposomes to increase the therapeutic activity of drugs has been proposed to be due to the ability of vesicular systems to enhance drug penetration (Betz, Aeppli, Menshutina, & Leuenberger, 2005), improve pharmacological properties (Sharma, Jain, & Vyas, 1994), control drug release (Fang, Leu, Chang, Lin, & Tsai, 2004) and serve as photoprotection system for drugs (Arsić & Vuleta, 2001). In many cases, the drugs of liposomal formulation have been used in recent years, with at least additive increases in therapeutic outcomes for the combinations compared to individual therapies.

In this study, the results showed that GLPL had significantly enhanced proliferation of the splenic lymphocyte of mice both in single stimulation and synergistical stimulation with PHA or LPS, which indicated immunological activity of GLP was enhanced by liposome delivery system and provides the basis for future research.

4. Conclusion

Preparation of liposomes using RSM was found to be well suited and sound approach to obtain optimal liposomal formulation of GLP. The optimal preparation conditions for the GLPL were as follows: soybean phosphatide to cholesterol ratio of 11:1, soybean phosphatide to tween 80 ratio of 10.5:1 and ultrasonic time (min) of 11. Under these conditions, the GLPL were small homogenous vesicles and the experimental EE was 71.43 ± 0.49%, which was close to the predicted value (Table 4). From the results of GLPL on splenic lymphocyte proliferation test in vitro, it could be concluded that stimulation on splenic lymphocyte proliferation of GLPL was significantly improved after encapsulation with liposomes. In brief, these findings indicated that the immunological enhancement of GLP was significantly enhanced after encapsulation with liposome, which provides the basis for further intensive study.

Acknowledgments

The project was supported by National Natural Science Foundation of China (Grant no. 31372472), Special Fund for Agro-scientific Research in the Public Interest (Grant no. 201303046, 201403051), Independent Innovation of Agricultural Sciences Program of Jiangsu Province (CX(12)5063), and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We are grateful to all other staffs in the Institute of Traditional Chinese Veterinary Medicine of Nanjing Agricultural University for their assistance in the experiments.

References

Allen, T. M., & Cullis, P. R. (2013). Liposomal drug delivery systems: From concept to clinical applications. *Advanced Drug Delivery Reviews*, *65*(1), 36–48.

Arsić, I., & Vuleta, G. (2001). Influence of liposomes on the stability of vitamin A incorporated in polyacrylate hydrogel. *International Journal of Cosmetic Science*, *21*(4), 219–225.

Bao, X.-F., Wang, X.-S., Dong, Q., Fang, J.-N., & Li, X.-Y. (2002). Structural features of immunologically active polysaccharides from *Ganoderma lucidum*. *Phytochemistry*, *59*(2), 175–181.

Berovič, M., Habijanič, J., Zore, I., Wraber, B., Hodžar, D., Boh, B., et al. (2003). Submerged cultivation of *Ganoderma lucidum* biomass and immunostimulatory effects of fungal polysaccharides. *Journal of Biotechnology*, *103*(1), 77–86.

Betz, G., Aeppli, A., Menshutina, N., & Leuenberger, H. (2005). In vivo comparison of various liposome formulations for cosmetic application. *International Journal of Pharmaceutics*, *296*(1), 44–54.

Fang, J.-Y., Leu, Y.-L., Chang, C.-C., Lin, C.-H., & Tsai, Y.-H. (2004). Lipid nano/submicron emulsions as vehicles for topical flurbiprofen delivery. *Drug Delivery*, *11*(2), 97–105.

Ghadiri, M., Fatemi, S., Vatanara, A., Doroud, D., Najafabadi, A. R., Darabi, M., et al. (2012). Loading hydrophilic drug in solid lipid media as nanoparticles: Statistical modeling of entrapment efficiency and particle size. *International Journal of Pharmaceutics*, *424*(1), 128–137.

Grazia Calvagno, M., Celia, C., Paolino, D., Cosco, D., Iannone, M., Castelli, F., et al. (2007). Effects of lipid composition and preparation conditions on physical-chemical properties, technological parameters and in vitro biological activity of gemcitabine-loaded liposomes. *Current Drug Delivery*, *4*(1), 89–101.

Gruber, S. A., Venkataram, S., Canafax, D. M., Cipolle, R. J., Bowers, L., Elsberry, D., et al. (1989). Liposomal formulation eliminates acute toxicity and pump incompatibility of parenteral cyclosporine. *Pharmaceutical Research*, *6*(7), 601–607.

Gunter, K., Gunter, T., Jarkowski, A., & Rosier, R. (1982). A method of resuspending small vesicles separated from suspension by protamine aggregation and centrifugation. *Analytical Biochemistry*, *120*(1), 113–124.

Gupta, V. K., Assmus, M. W., Beckert, T. E., & Price, J. C. (2001). A novel pH-and time-based multi-unit potential colonic drug delivery system. II. Optimization of multiple response variables. *International Journal of Pharmaceutics*, *213*(1), 93–102.

Huang, Y., Wu, C., Liu, Z., Hu, Y., Shi, C., Yu, Y., et al. (2014). Optimization on preparation conditions of *Rehmannia glutinosa* polysaccharide liposome and its immunological activity. *Carbohydrate Polymers*.

Ko, J., Park, H., Park, Y., Hwang, S., & Park, J. (2003). Chitosan microparticle preparation for controlled drug release by response surface methodology. *Journal of Microencapsulation*, *20*(6), 791–797.

Konigsberg, P., Godtel, R., Kissel, T., & Richer, L. (1998). The development of IL-2 conjugated liposomes for therapeutic purposes. *Biochimica et Biophysica Acta (BBA)—Biomembranes*, *1370*(2), 243–251.

Lin, K.-I., Kao, Y.-Y., Kuo, H.-K., Yang, W.-B., Chou, A., Lin, H.-H., et al. (2006). Reishi polysaccharides induce immunoglobulin production through the TLR4/TLR2-mediated induction of transcription factor Blimp-1. *Journal of Biological Chemistry*, *281*(34), 24111–24123.

Lin, Z. (2005). Cellular and molecular mechanisms of immuno-modulation by *Ganoderma lucidum*. *Journal of Pharmacological Sciences*, *99*(2), 144.

Minato, K.-I., Kawakami, S., Nomura, K., Tsuchida, H., & Mizuno, M. (2004). An exo β-1, 3-glucanase synthesized de novo degrades lentinan during storage of *Lentinule edodes* and diminishes immunomodulating activity of the mushroom. *Carbohydrate polymers*, *56*(3), 279–286.

Muralidhar, R., Chirumamila, R., Marchant, R., & Nigam, P. (2001). A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources. *Biochemical Engineering Journal*, *9*(1), 17–22.

Murthy, M., Swaminathan, T., Rakshit, S., & Kosugi, Y. (2000). Statistical optimization of lipase catalyzed hydrolysis of methyloleate by response surface methodology. *Bioprocess Engineering*, *22*(1), 35–39.

Paterson, R. R. M. (2006). Ganoderma—A therapeutic fungal biofactory. *Phytochemistry*, *67*(18), 1985–2001.

Sharma, B., Jain, S., & Vyas, S. (1994). Topical liposome system bearing local anaesthetic lignocaine: Preparation and evaluation. *Journal of Microencapsulation*, *11*(3), 279–286.

Sinha, K., Chowdhury, S., Saha, P. D., & Datta, S. (2013). Modeling of microwave-assisted extraction of natural dye from seeds of *Bixa orellana* (Annatto) using response surface methodology (RSM) and artificial neural network (ANN). *Industrial Crops and Products*, *41*, 165–171.

Wang, R., Chen, P., Jia, F., Tang, J., & Ma, F. (2012). Optimization of polysaccharides from *Panax japonicus* CA Meyer by RSM and its anti-oxidant activity. *International Journal of Biological Macromolecules*, *50*(2), 331–336.

Watson, D. S., Endsley, A. N., & Huang, L. (2012). Design considerations for liposomal vaccines: Influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. *Vaccine*, *30*(13), 2256–2272.

Xu, H., Sun, L.-P., Shi, Y.-Z., Wu, Y.-H., Zhang, B., & Zhao, D.-Q. (2008). Optimization of cultivation conditions for extracellular polysaccharide and mycelium biomass by *Morchella esculenta* As51620. *Biochemical Engineering Journal*, *39*(1), 66–73.

Xu, J., Liu, W., Yao, W., Pang, X., Yin, D., & Gao, X. (2009). Carboxymethylation of a polysaccharide extracted from *Ganoderma lucidum* enhances its antioxidant activities in vitro. *Carbohydrate Polymers*, *78*(2), 227–323.

Xu, J., Zhang, Y., & Wang, Y. (2004). Application of liposomes in traditional Chinese medicine research. *Qilu Pharmaceutical Affairs*, *9*, 40–41.

Yang, S., Chen, J., Zhao, D., Han, D., & Chen, X. (2012). Comparative study on preparative methods of DC-Chol/DOPE liposomes and formulation optimization by determining encapsulation efficiency. *International Journal of Pharmaceutics*, *434*(1), 155–160.

Yolmeh, M., Najafi, M. B. H., Farhoosh, R., & Hosseini, F. (2014). Optimization of ultrasound-assisted extraction of natural pigment from annatto seeds by response surface methodology (RSM). *Food Chemistry*.

Yu, H., Teng, L., Meng, Q., Li, Y., Sun, X., Lu, J., et al. (2013). Development of liposomal Ginsenoside Rg3: Formulation optimization and evaluation of its anticancer effects. *International Journal of Pharmaceutics*, *450*(1), 250–258.

Yu, X., Zhang, K., & Zheng, X. (2003). Study on the determination of Aloe oligosaccharides. *Acta Nutrimenta Sinica*, *25*, 149–151.

Yue, G. G., Fung, K. P., Leung, P. C., & Lau, C. (2008). Comparative studies on the immunomodulatory and antitumor activities of the different parts of fruiting body of *Ganoderma lucidum* and Ganoderma spores. *Phytotherapy Research*, *22*(10), 1282–1291.

Zhang, P., Ding, R., Jiang, S., Ji, L., Pan, M., Liu, L., et al. (2014). The adjuvanticity of *Ganoderma lucidum* polysaccharide for Newcastle disease vaccine. *International Journal of Biological Macromolecules*, *65*, 431–435.

Zhou, X., Lin, J., Yin, Y., Zhao, J., Sun, X., & Tang, K. (2007). Ganodermataceae: Natural products and their related pharmacological functions. *The American Journal of Chinese Medicine*, *35*(04), 559–574.