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Polysaccharides of *Atractylodes macrocephala* Koidz-loaded nanostructured lipid carriers: Optimization on conditions by RSM and immunological activity *in vitro*

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ABSTRACT

The main purpose of this study was the preparation, optimization, characterization, and *in vitro* immunological activity evaluation of the polysaccharides of *Atractylodes macrocephala* Koidz-loaded nanostructured lipid carriers (PAMK-NLC). Both the blank NLC and PAMK-NLC were prepared using melt-emulsification and ultrasonication techniques. The optimization of preparation conditions were a stearic acid to caprylic/capric triglyceride mass ratio of 2:1, a poloxamer 188 to soybean lecithin mass ratio of 2:1, and an ultrasound time of 12 min; these parameters were identified using response surface methodology (RSM). Under these conditions, the encapsulation efficiency was around 76.85% and the polydispersity index was around 0.281, indicating a good dimensional distribution. Furthermore, the potential of PAMK-NLC to enhance immunological activity *in vitro* was investigated in murine splenic lymphocytes. The results showed that PAMK-NLC could significantly stimulate lymphocyte proliferation and increase the ratio of $CD4^+$ to $CD8^+$ T cells. In conclusion, these findings indicated that PAMK-NLC possessed an efficient immunological activity, and that NLC is suitable for the delivery and protection of PAMK in clinical use.

1. Introduction

Atractylodes macrocephala Koidz (Baizhu in Chinese), a traditional Chinese herbal medicine, has both medicinal and food uses [1]. The rhizome of *Atractylodes macrocephala* Koidz contains mainly medicinal components (especially polysaccharides), which have many pharmacological effects such as invigorating the spleen, immune stimulation [2], antioxidant activity [3], and so on. Polysaccharides of *A. macrocephala* Koidz (PAMK) include glucose, xylose, ribose, mannose, rhamnose, and galactose; and, in particular, β-glucose, β-galactose, and β-galacturonic acid. The backbone is 1 → 6 linked glucose [4,5]. There are reports that PAMK could significantly improve disordered intestinal flora [6] and regulate the immune function by changing some cytokines to proper levels [7]. However, there are some negative characteristics of PAMK, for example, it is rapidly metabolized, its range of action is not concentrated, and large dosages are required in clinical practice. Given the defects of PAMK in clinical use, further studies are required to investigate its bioactivity, improve its bioavailability, and increase its range of applications in drug delivery systems.

Nanostructured lipid carriers (NLC) are one of the most frequently studied lipid-based formulations, and have shown great potential as delivery systems for a variety of drugs [8]. NLC have high drug-loading capacities for both lipophilic and hydrophilic drugs [9,10]. NLC, in which the lipid phase consists of a mixture of solid lipid and liquid lipid at room temperature [11,12], were developed from solid lipid nanoparticles (SLN) with some modifications in the late 1990s, as a way to overcome the limited drug loading and drug leakage during storage that occurred with SLN [13]. The mixing of liquid and solid lipid results in imperfect crystalline structures that can carry more drugs [14]. In general, NLC systems have the merits of SLN, including low cost of supplies, convenient preparation and enhanced biocompatibility [15], as well as improving on their shortcomings [16]. Consequently, in this

Abbreviations: PAMK, polysaccharides of *Atractylodes macrocephala* Koidz; NLC, nanostructured lipid carriers; PAMK-NLC, polysaccharides of *Atractylodes macrocephala* Koidz-loaded nanostructured lipid carriers; SA, Stearic acid; CCT, Caprylic/capric triglyceride; F68, Poloxamer 188; SL, Soybean lecithin; OP, oil phase; WP, water phase; RSM, response surface methodology; LPS, lipopolysaccharide; PHA, phytohemagglutinin; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; EE, encapsulation efficiency; BBD, Box-Behnken design; ANOVA, analysis of variance; PDI, polydispersity index

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study, NLC are used to encapsulate PAMK, in order to increase its drug-loading efficiency and enhance its bioactivity.

Response surface methodology (RSM), as an effective statistical method, is used here to optimize the preparation conditions of PAMK-NLC. In recent years, this method has been successfully used to determine the effects of multiple variables and to optimize biochemical processes [17]. Compared with other methods, RSM has the advantages of simplifying the complexity of the experimental trials in which multiple parameters and their interactions need to be evaluated, as well as allowing for more efficient and simple arrangement and interpretation of experiments [18–20].

In this study, we demonstrate a facile approach to encapsulate PAMK in NLC, and further optimize the preparation conditions of PAMK-NLC by RSM. Lymphocytes are known to have an important role in the immune system, where they not only initiate innate immune responses, but also serve as effector cells in inflammatory reactions and the anti-infection response process [21]. In order to investigate the possible immunomodulatory effects of PAMK-NLC *in vitro*, the proliferation of spleen lymphocytes and the expression of $CD4^+$ and $CD8^+$ T lymphocytes were evaluated by MTT assay and flow cytometry, respectively.

2. Materials and methods

2.1. Materials

PAMK (80%), caprylic/capric triglyceride (CCT), poloxamer 188 (F68), and Sephadex G-50 were purchased from Shanghai Yuanye Biotechnology Co., Ltd. Stearic acid (SA) was from Sigma (China), and lecithin from soybean (SL) was from Aladdin (China). RPMI-1640 (GIBCO) was supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin. Red Cell Lysis Solution was purchased from Biosharp. MTT (Biosharp) was dissolved into 5 mg/mL with phosphate-buffered saline (PBS) in a dark bottle. Lipopolysaccharide (LPS) and phytohemagglutinin (PHA) were purchased from Sigma and stored at −20 °C. Ethanol and dimethyl sulfoxide (DMSO) were purchased from Nanjing Chemical Reagent Ltd.

2.2. Preparation of PAMK-NLC

The formulations of PAMK-NLC were produced by melt-emulsification and ultra-sonication techniques [22]. In brief, the desired amounts of SA, CCT, and SL (2:1:1) were dissolved in the minimum amount of ethanol and heated to 75 °C with moderate stirring to make the oil phase (OP) lucid and uniform. Meanwhile, the water phase (WP) consisted of surfactant F68 dispersed in deionized water, together with the desired concentration of PAMK solution which was heated to the same temperature. Thereafter, the WP was poured into the OP under magnetic stirring at 1000 rpm in a hot water bath for 30 min. The obtained pre-emulsion was homogenized by a probe-ultrasonic cell disruptor for 12 min (2 s on, 3 s off) at 100 W and dispersed rapidly into deionized water (0–2 °C) in an ice bath with stirring at 1000 rpm for 2 h in order to solidify particles and to remove volatile residual organic solvents. The same procedure, without adding PAMK to the water phase, was used to prepare blank NLC. The particles obtained from these two procedures were stored at 4 °C.

2.3. Encapsulation efficiency

The PAMK encapsulation efficiency (EE) of the formulations was determined using a mini-column centrifugation method [23]. After centrifugation, the eluent was collected, and the content of encapsulated PAMK was quantitatively determined by phenol-sulfuric acid spectrophotometry at 490 nm. The total PAMK was assessed with the same method. All analyses were conducted in triplicate. The EE was calculated with the equation: EE% = (Encapsulated PAMK/Total PAMK) × 100% (1).

Table 1
Independent variables and their levels used in the response surface design.

Independent variables	Symbol	Factor level		
		−1	0	1
Ratio of SA to CCT (w/w)	X_1	1	2	3
Ratio of F68 to SL (w/w)	X_2	1	2	3
Ultrasound time (min)	X_3	8	12	16

2.4. Single-factor experimental design

PAMK-NLC were prepared by the method described in section 2.2. The EE was considered as the evaluation indicator in this experiment. Four factors (mass ratio of SA to CCT, mass ratio of F68 to SL, volume ratio of WP to OP, and ultrasound time) potentially affecting the EE of PAMK-NLC were assessed by a single-factor design, in which one factor was changed in each experiment while the other factors were kept constant.

2.5. Optimization of PAMK-NLC preparative conditions by RSM

After the preliminary single-factor experiment, the preparation conditions for PAMK-NLC were optimized by RSM to obtain the best process parameters. A Box–Behnken design (BBD) was used with three independent variables, namely, mass ratio of SA to CCT (X_1), mass ratio of F68 to SL (X_2) and ultrasound time (X_3). Each variable was coded at three levels: −1, 0 and + 1, which are given in Table 1. The complete quadratic equation used for this model was as follows:

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

where Y is the response function, X_i and X_j are the levels of the independent variables ($i \neq j$), A_0 is constant, and A_i, A_{ii}, and A_{ij} are coefficients estimated by the model, representing the linear, quadratic, and cross-product effects of the X_1, X_2, and X_3 factors on the response, respectively.

2.6. Characterization

The mean particle size and polydispersity index (PDI) values of the formulations were determined by dynamic light scattering (DLS) using a laser particle size analyzer (Malvern Instruments, UK) at 25 °C with an angle detection of 90°. To avoid multiple scattering events, the sample was diluted with deionized water before measurement until the proper particle concentration was achieved. Each measurement was repeated three times. In order to observe the surface morphology of PAMK-NLC, transmission electron microscopy (TEM; Model Tecnai12, Philips Co., Ltd.) was used.

2.7. In vitro splenic lymphocyte study

2.7.1. Cytotoxicity assay

Splenic lymphocytes were harvested from untreated ICR mice (5 weeks of age), based on previously described methods [24] with some modifications. Briefly, the spleens were collected under aseptic conditions, then gently minced and passed through a disposable sterile cell screen. In order to remove the red blood cells, Red Cell Lysis Solution was added to the cell suspension for 3 min and the splenocytes were washed twice. Finally, the pelleted cells were re-suspended and diluted to 2.5×10^6 cells/mL with RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin solution. The cytotoxicities of NLC and PAMK-NLC were evaluated in lymphocytes using the MTT assay [25] described previously. The absorbance of each well