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Evaluation of optimum conditions for *Achyranthes bidentata* polysaccharides encapsulated in cubosomes and immunological activity *in vitro*

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ABSTRACT

Cubosomes, as biocompatible carriers in drug delivery systems, consist of curved bicontinuous lipid bilayers. With a honeycombed structure divided into two internal aqueous channels, cubosomes could be used for many bioactive ingredients. *Achyranthes bidentata* polysaccharides (ABPs) are isolated from the roots of *Achyranthes bidentata*, used in Chinese herbal medicine, and present a noticeable effect as an immunomodulator. This study investigates the optimal preparation of combined cubosome-ABP (Cub-ABP) nanoparticles using response surface methodology and explores their characteristics and stability. The encapsulation efficiency of optimized Cub-ABPs was 72.59%. *In-vitro* stability studies demonstrated the stability of Cub-ABPs and cubosome nanoparticles without ABPs; both were stable for up to 25 days. Safe concentrations of Cub-ABPs and cubosome nanoparticles without ABPs are 104.06 μg/mL and 208.13 μg/mL with comparatively low cytotoxicity against lymphocytes. Moreover, the feasible immunomodulatory effects of Cub-ABPs were determined by evaluating their proliferation and change of $CD4^+/CD8^+$ ratio on splenic lymphocytes *in vitro*. Proliferation and flow cytometry studies revealed that, compared with free ABPs and blank cubosomes, Cub-ABPs proved more effective in promoting lymphocyte proliferation and in triggering the transformation of T-lymphocytes into T_h-cells.

1. Introduction

Nanotechnology plays a decisive role in the contemporary medical field, especially in drug delivery. About 20 years ago, the liquid crystalline cubic phase of hydrated lipids was developed by Swedish scientists to form particles called cubosomes; since then, the use of cubosomes as a form of nanocarrier has burgeoned [1]. These amphiphilic molecules, which are used to protect loaded materials, are well received in drug delivery systems. They often have highly organized structures and self-assembly ability under particular conditions [2–4]. This study has focused on a liquid-crystal-forming lipid based on glyceryl monooleate. The internal structure of the cubosome nanoparticles, which consists of a lipid domain, a water channel, and an interfacial region, can be used to encapsulate hydrophilic, lipophilic, and protein drugs [5–7].

Chinese herbal medicines have been used for centuries to maintain human and animal health in China; many clinical studies have indicated that herbal medicines are extremely effective for treating medical disorders in a wide variety of areas [8–10]. *Achyranthes bidentata*, originally from Shen Nong Ben Cao Jing, which is widely acknowledged for its function of invigorating livers and kidneys, as well as strengthening muscles and bones [11], is used to invigorate blood and break blood stasis, and is extensively used in the treatment of osteoarthritis [12–14]. There are many interrelationships between plant polysaccharides and immune systems, both innate and adaptive [15,16]. *Achyranthes bidentata* polysaccharides (ABPs) [17,18] are the active components isolated from *Radix Achyranthis bidentatae*, or the root of *Achyranthes bidentata*, and are reported to

Abbreviations: ABP, *achyranthes bidentata* polysaccharide; ANOVA, analysis of variance; Cub, cubosome; Cub-ABP, cubosome loaded with *Achyranthes bidentata* polysaccharide; DMEM, Dulbecco's Modified Eagle's Medium; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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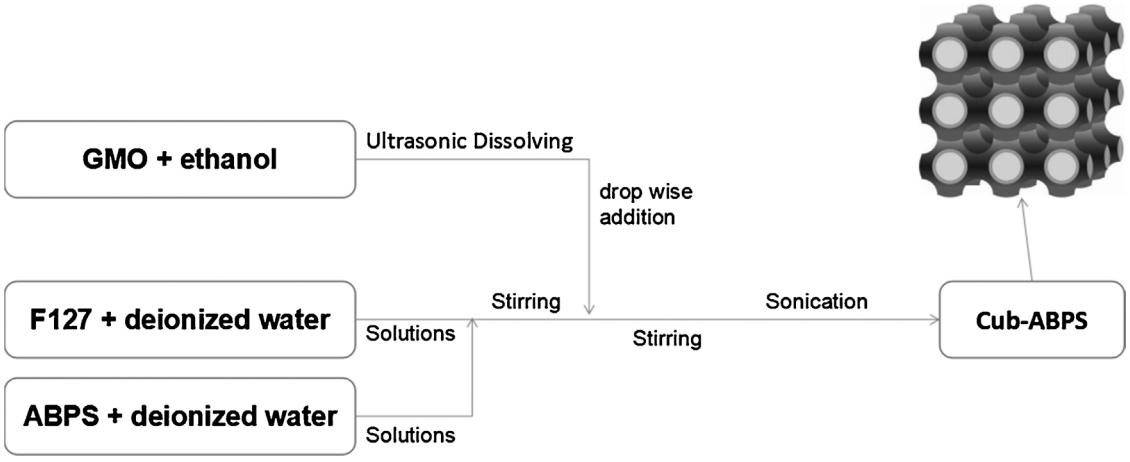

Fig. 1. The schematic presentation of the fabrication process of Cub-ABPS.

exert a wide spectrum of immunomodulatory effects on the cells of the immune system [16,19–21]. In recent years, many studies have been carried out on the isolation, purification, and structural characterization of ABPs [17,18,22,23]. However, a few negative characteristics of polysaccharides hinder their clinical application; for instance, their small molecular weight and hydrophilicity, leading to easy degradation and a short half-life *in vivo*. Hence, there are few reports on the application of ABPs in vaccines.

Accordingly, we hypothesized that a combination of cubosomes and ABPs would deliver better immune-regulation effects by increasing bioavailability and prolonging circulation time. In this study ABPs were encapsulated in cubosomes and the bioactivity of the resulting combination was evaluated. Encapsulation efficiency was used as an index. Response surface methodology was applied to optimize the Cub-ABP preparation scheme. The characteristics of Cub-ABPs were determined using small angle X-ray scattering and transmission electron microscopy; a laser particle-size analyzer was used to determine the size of the cubosome nanoparticles. The stability of both Cub-ABPs and Cub nanoparticles without ABPs was determined *in vitro*. Moreover, the feasible immunomodulatory effects of the Cub-ABPs were determined by evaluating their proliferative effect and changes of $CD4^+/CD8^+$ ratio on splenic lymphocytes *in vitro*. Through these experiments, the optimization of particle preparation has been achieved, as a basis for a next step study for their use as an immunomodulatory adjuvant.

2. Materials and methods

2.1. Materials

The ABP (98%, S25817) was manufactured by Shanghai Yuanye Biotechnology Co., Ltd. Glyceryl monooleate (99%) was obtained from Shanghai Macklin Biochemical Co. Ltd. Pluronic F127 was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sephadex G-50 was purchased from Shanghai Yuanye Biotechnology Co., Ltd. RPMI-1640, fetal bovine serum, and phosphate-buffered saline, acquired from Thermo Fisher Scientific (Waltham, MA, USA), were used for the stability study and as diluents for the drug and nanospheres. RPMI-1640 and Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, penicillin 100 IU/mL, and streptomycin 100 IU/mL, were used for cell culture. Phytohemagglutinin and lipopolysaccharide were procured from Sigma-Aldrich Co. Red Blood Cell Lysis Buffer, MTT, and Cell Counting Kit-8 were procured from Biosharp Biotechnology Co. (Hefei, Anhui, China). A Mouse IL-4 ELISA Kit and Mouse IFN-gamma ELISA Kit were supplied by MultiSciences Inc. (Hangzhou, Jiangsu, China). Anti-CD3e-PE-Cyanine5, anti-CD4-FITC, and anti-CD8a-PE antibodies were supplied by eBioscience Inc. (San Diego, CA, USA). Dimethyl sulfoxide, anhydrous ethanol, concentrated sulfuric acid, and other chemicals were of analytical grade and used without further purification.

2.2. Preparation and evaluation

2.2.1. Preparation of cubosomes

Briefly, ABP solution (dissolved in deionized water) was added to an aqueous solution of F127 (dissolved in deionized water at 70 °C) while stirring, until the temperature of the dispersion system fell to about 60 °C. Monooleate dissolved in anhydrous ethanol was added dropwise to the dispersion at a controlled temperature (60 °C). The dispersion was equilibrated and volatilized by magnetic stirring at 60 °C for longer than 1 h to remove the highly volatile organic solvent (ethanol) before ultrasonic emulsification and autoclaving. The final product was homogenized by ultrasonication (MisonixXL2000, Misonix Inc., Nanjing, China). Cubosomes without added ABPs (Cubs) were prepared in the same way, except without the ABP. All samples were stored in glass vials at room temperature and protected from light. The process is illustrated in Fig. 1.

2.2.2. Encapsulation efficiency

The quantity of ABPs encapsulated in Cub-ABPs nanoparticles was determined by a modified microcolumn centrifugation method and ultraviolet spectrophotometry [24,25]. Sephadex G-50 powder was soaked in 0.9% NaCl for 12 h until completely swollen. A filter pad was inserted into a 2 mL syringe without a piston. The swollen Sephadex G-50 was carefully added dropwise to the syringe, ensuring that all the bubbles were removed. The columns were put in 10 mL centrifuge tubes and centrifuged at 2000 rpm for 10 min to remove excess water. Then 0.5 mL Cub-ABPs were slowly and carefully added to the prepared columns and centrifuged at 1000 rpm for 8 min. The unencapsulated ABP was adsorbed to the gel inside the columns, while the nanoparticles were collected for analysis. Aliquots of 0.5 mL deionized water were added dropwise to the tops of the columns, which were then centrifuged at 2000 rpm for 5 min; this was repeated six times. All of the free ABP was eluted from the column. The accurate content of encapsulated ABPs was quantified using the improved vitriol method; one-sixth of the quantity of ABPs was encapsulated in 0.5 mL Cub-ABPs.

The formula for Cub-ABP encapsulation efficiency is:

$$\text{Encapsulationefficiency}(\%) = (1 - C_f/C_t) \times 100\%,$$