Chitosan microspheres loading whole cell protein antigen of *Helicobacter pylori*: preparation and *in vitro* release characteristics

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Abstract

BACKGROUND: Studies on encapsulated whole cell protein antigen of *Helicobacter pylori* are still at the exploration stage. There is limited literature concerning the preparation process and *in vitro* release characteristics of chitosan microspheres encapsulated with whole cell protein antigen of *Helicobacter pylori*.

OBJECTIVE: To explore the preparation process and *in vitro* release characteristics of chitosan microspheres encapsulating whole cell protein antigen of *Helicobacter pylori*.

METHODS: Precipitation method was used to prepare chitosan microspheres, and the best preparation process, matching and encapsulation time were screened. Under electron microscope, the morphology and particle size of microspheres were observed. Chitosan microspheres were used to encapsulate *Helicobacter pylori* whole cell protein antigen, and BCA method was used to determine encapsulation efficiency, encapsulation content and release efficiency *in vitro* of Helicobacter pylori whole cell protein antigen.

RESULTS AND CONCLUSION: Final concentration of 1% glacial acetic acid, sodium sulfate as crosslinking agent, pH=5.0, with no pulverization when the crosslinking agent was added was the best preparation process for chitosan microspheres. Electron microscopy showed the smooth surface morphology of microspheres with roundness and good dispersion, and the majority of the microspheres were 1–5 μm in diameter. The encapsulation efficiency of *Helicobacter pylori* whole cell protein antigen microspheres was 80.4%, the encapsulated amount was 16.4%, and total 48-hour release rate was 19.4%. *Helicobacter pylori* whole cell protein antigen microspheres showed an overall slow release status. Chitosan microspheres show good encapsulation efficiency and amount of *Helicobacter pylori* whole cell protein antigen, and Helicobacter pylori total bacteria protein antigen microspheres show an overall slow release status.

Subject headings: Tissue Engineering; Biocompatible Materials; Chitosan; Vaccines

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INTRODUCTION

Helicobacter pylori (Hp) is the main pathogenic bacterium for digestive system diseases and its drug resistance is significantly increased[1-2]. However, Hp eradication treatment with medicine is difficult, and how to prepare anti-Hp vaccine has become the research focus. Microspheres, due to its advantage on targeted drug release, have received diverse attentions from all over the world. It has proved that microspheres can enhance the immune function[3]. There are numbers of literatures about microsphere preparation materials. How to choose degradable materials with good biocompatibility, simple process and releasing characteristic is the study hotspot. Chitosan, a natural alkaline polysaccharide, shows good biocompatibility, nontoxicity and degradability[4-5]. In addition, preparation of microspheres using chitosan can avoid the influence of high temperature on protein medicine and decrease the loss of proteins. Preparing protein microspheres with chitosan not only exhibits controlled-release effect, but also enhance bioavailability through directly attaching to the intestinal mucosa. In the present study, chitosan was used as a carrier to prepare microspheres encapsulating Hp protein antigen, and its releasing properties *in vitro* were investigated, aiming at providing guidelines for preparing Hp vaccine.

MATERIALS AND METHODS

Design

Materiology experiments.

Time and setting

Experiments were completed at the laboratory

of the Medical College of Qingdao University from September 2012 to January 2015.

Materials

Reagents and instruments for preparation of chitosan microspheres loading whole cell protein antigen of Hp:

Instrument and reagent	Source
Chitosan	Weifang Haizhiyuan Biological Products Co., Ltd.
Hp standard strain	American Type Culture Collection (ATCC), USA
BCA method protein content detecting kit	Nanjing Kaiji Biological Technology Development Co., Ltd.
Sterility anticoagulants sheep whole blood	Nanjing Senjiabei Biological Technology Co., Ltd.
Campylobacter jejuni agar	Shanghai Yansheng Biochemical Regent Co., Ltd.
Tri-gas incubator	Tianjin Fuma Science and Technology Co., Ltd.
High speed centrifuge	Beckman, USA
S4800 scanning electron microscopy	Hitachi, Japan
Melvin particle size analyzer	MasterSizer 2000, England
Magnetic stirrers	Shanghai Renhe Scientific Instrument Co., Ltd.
Microplate reader	Bio-rad680, America

Methods

Hp culture method

Hp standard strain was inoculated on campylobacter jejuni agar containing sheep whole blood. Culture condition: 5% O_2, 10% CO_2, 85% N_2 and 37 ℃. After culturing for 2 days, the cultured bacteria were qualified, washed-out, and the absorbance value at 660 nm was determined.

Hp whole cell protein antigen preparation

The cultured Hp standard strain was eluted with PBS (0.01 mol/L) for three times, followed by smashed with ultrasound and centrifuged (4 ℃, 30 minutes). The supernatant was collected and the protein content was investigated with BCA method and saved in a-20 ℃ freezer.

Preparation optimization of chitosan microspheres

Chitosan was dissolved in glacial acetic acid, and the Tween-80 (1%) as dispersing agent, sodium sulfate or sodium tripolyphosphate as cross-linking agent was added to prepare chitosan microsphere precipitate. In this experiment, 1% or 2% glacial acetic acid, sodium sulfate or sodium tripolyphosphate was used as cross-linking agent. The detailed 16 kinds of preparation schemes are listed in **Table 1**. The prepared microspheres were observed using scanning electron microscopy.

Preparation of chitosan microspheres after optimization

The optimum preparation conditions were confirmed according to scanning electron microscopy results. 1.5 mg chitosan was dissolved in 500 mL distilled water (at a concentration of 3%), which was stirred using magnetic stirrers (500 r/min), and 3 mL of 1% glacial acetic acid was added. When chitosan was totally dissolved, 5 mL Tween-80 (1%) was added to adjust pH to 5.0 and 2% sodium sulfate was added drop by drop until the precipitate occurred, and the stirred time was 2 hours. The resulted product was centrifuged (2 500 r/min×10 minutes) for three times and the precipitate was collected, freeze-dried, and save at 4 ℃.

Table 1 Preparation optimization of chitosan microspheres

Factor	Level	
Glacial acetic acid	1%	2%
Cross-linking agent	sodium sulfate	sodium tripolyphosphate
pH	5.0	5.5
Smashed with ultrasound	Yes	No

Optimization of microsphere optimum ratio and encapsulating time

Hp whole cell protein antigen and chitosan microspheres at fixed ratio was dissolved in acetate buffer (10 mL, 0.1 mol/L, pH 6.0) and stirred for 24 hours at room temperature. The encapsulation efficiency of resulted products was determined and the optimum ratio of Hp whole cell protein antigen to chitosan microspheres was obtained (**Table 2**). The optimum encapsulating time was investigated by determining the encapsulation efficiency at 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24 hours using the optimum preparation method. Encapsulation efficiency=(total protein content-reminded protein content)/total protein content. Encapsulation content=(total protein content-residual protein content)/chitosan microspheres content.

Table 2 Preparation optimization of chitosan microspheres

Hp whole cell protein antigen: chitosan microspheres	Hp whole cell protein antigen (mg)	Chitosan microspheres (mg)
1:1	50	50
1:2.5	50	125
1:5	50	250
1:7.5	50	375

Preparation of Hp whole cell protein antigen microspheres

According to the optimum ratio and encapsulating time mentioned above, 500 mg chitosan microspheres and 100 mg Hp whole cell protein antigen were dissolved in acetate buffer (50 mL, 0.1 mol/L, pH 6.0) and stirred for 2 hours at room temperature, centrifuged (1 000 r/min× 20 minutes), to afford Hp whole cell protein antigen microspheres. 50 mL PBS (pH=6.8) was added to the precipitate, stirred, and 2 mL solution was collected at 1, 3, 6, 9, 12, 24, 48 hours, respectively, to determine the

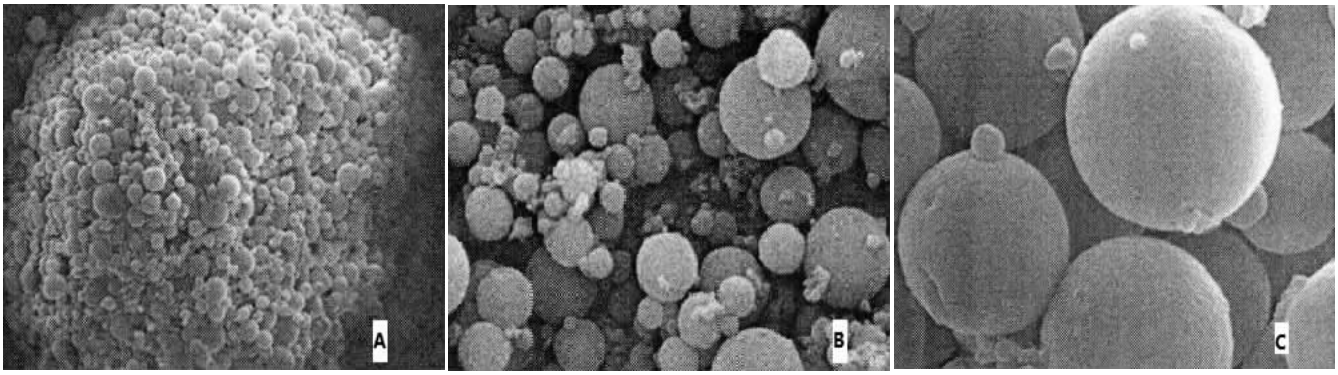

Figure 1 Scanning electron microscopy of *Helicobacter pylori* whole cell protein antigen microspheres
Note: (A)×1 500; (B)×3 000; (C)×10 000.

protein content with BCA method. Release efficiency=release content in supernatant/(total encapsulation content–residual protein content). Blank chitosan microspheres were used as controls.

Main outcome measures
Morphological characteristics, particle size, encapsulation efficiency, encapsulation content, release efficiency.

RESULTS

Optimum preparation condition and morphological characteristics
The optimum preparation condition was 1% glacial acetic acid, sodium sulfate as cross-linking agent, pH=5.0, and non-smashing process. Hp whole cell protein antigen microspheres were smooth and round spheres with good dispersibility, and the particle size was 1.0–5.0 μm, under scanning electron microscopy, shown in **Figure 1**.

Optimum ratio and encapsulating time optimization for microspheres
The experiment results are listed in **Table 2**, from which the optimum ratio of Hp whole cell protein antigen to chitosan microspheres was calculated to be 5:1. The encapsulation efficiency at 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24 hours was 67.4%, 53.7%, 77.8%, 54.9%, 58.3%, 69.1%, 66.4%, 61.5%, 71.3% and 44.6%, respectively. Therefore, the optimum ratio was 5:1, and the optimum encapsulating time was 3 hours.

Encapsulation efficiency, encapsulation content and release efficiency *in vitro*
The encapsulation efficiency and encapsulation content of Hp whole cell protein antigen microspheres was 80.4% and 16.4%, respectively. The release rate at 1, 3, 6, 9, 12, 24, and 48 hours was 17.8%, 18.2%, 18.4%, 18.7%, 18.9%, 19.2% and 19.4%, respectively, indicating that Hp whole cell protein antigen microspheres had controlled release effect. No protein was detected in the supernatant of control group, showing that blank microspheres exerted no effect on protein release *in vitro* (**Figure 2**).

DISCUSSION

Using chitosan as the carrier can avoid the high temperature, organic environment, and decrease the loss of protein. Moreover, chitosan, as natural macromolecular

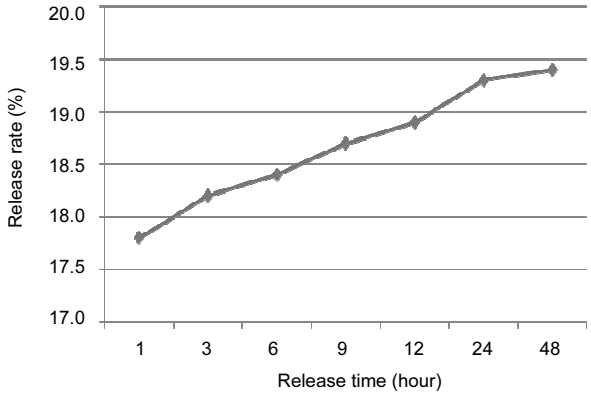

Figure 2 Release rate of *Helicobacter pylori* whole mycoprotein antigen in each period
Note: Blank microspheres exerted no effect on the *in vitro* release of *Helicobacter pylori* whole cell protein antigen.

substance, exhibit biodegradable and nontoxic properties, which provides a new idea for microspheres preparation. Li *et al* [6] reported that chitosan could encapsulate a large amount of antigens and showed controlled-release abilities, and enhanced the bioavailability, prolonged the residence time at the mucous membrane by adhesive action with epithelial cells. Particle size of microspheres, however, is tightly correlated with distribution *in vivo*, for example, microspheres with a particle size of less than 5 μm mainly distribute in nodi lymphatici mesenterici, spleen and macrophage in the blood system; microspheres with a particle size of over 5 μm mainly stay around the Peyer's patches, which are induced to produce sIgA antibodies[7-9]. Therefore, controlling the particle size of microspheres is good for playing its immune function extremely.

Nowadays, there are four methods to prepare microspheres: emulsification chemical-crossline method, solvent evaporation method, spray drying process and Berthold precipitation method, among which Berthold precipitation method is simple, without organic solvent, and

has low effect on encapsulated medicine, being helpful for maintaining bioactivities of protein. Liu *et al* [10] demonstrated that compared with the emulsification chemical-crossline method, the precipitation method could decrease the retention time at mouse stomach and increase the distribution at colon. For Hp whole cell protein antigen microspheres, increased distribution at colon could avoid the degradation under low pH circumstance at stomach. When preparing chitosan microspheres, chitosan acetate showed good solubility by adding glacial acetic acid, while decreased solubility by adding sulfate, which was beneficial to microspheres formation. Dilute acid was good for dissolving chitosan using Berthold precipitation method; however, chitosan could also degrade under acid circumstance. Thus, 1% acetic acid was used as acid, tween 80 as dispersing agent, and sodium sulfate as precipitator. During preparing microspheres, the concentration of acetic acid, pH, magnetic stirring, and dripping speed of sodium sulfate all had effect on microspheres quality. The resulted optimum preparation condition was 1% glacial acetic acid, sodium sulfate as cross-linking agent, and non-smashing process in this study, which was proved by electron microscope results. Through detecting the encapsulation efficiency of different ratio and encapsulation time, the optimum ratio was 5:1 and the optimum encapsulating time was 3 hours, under which the encapsulation efficiency was 70%–80%. The encapsulation efficiency of prepared microspheres under the optimum preparing method was 80.4%. Particle size of 80% of the prepared microspheres was 1.0–5.0 μm.

Drug release *in vitro* is an important index to evaluate the properties of microsphere. In this study, the release rate of Hp whole cell protein antigen microspheres at 1 hour was 17.8%, which may be due to the reason that part of the protein absorbed at the surface of microspheres directly diffused to solution and was proved by Wu *et al* [11]. Hu *et al* [12] prepared acyclovir chitosan microspheres with emulsification chemical crosslinking method and the release rate at 1 and 12 hours was 23.4%, and 87%, respectively. Drug release speed depended on the properties of chitosan microspheres, drug characteristic and circumstance[13-22]. Under 37 ℃, 50 mL PBS (pH=6.8), the release rate at 1, 24 and 48 hours was 17.8%, 19.2%, and 19.4%, respectively, demonstrating that chitosan microspheres had controlled-release effect. When the prepared Hp whole cell protein antigen microspheres were orally taken, it could be absorbed by the intestinal mucosa. And when the released antigen was phagocytosed by macrophage, the antigen could execute its immune function and prevent Hp infection function.

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壳聚糖微球包裹幽门螺杆菌全菌蛋白抗原的制备及体外释放性能

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文章亮点：
以壳聚糖用作载体，采用沉淀法来制备微球包裹幽门螺杆菌蛋白抗原，通过检测包裹率、包裹量和体外释放率，观察其体外释放性能。实验结果证实，此载体具有良好的包裹率和包裹量，幽门螺杆菌全菌蛋白抗原微球整体呈缓慢释放状态，以期为制备幽门螺杆菌疫苗提供依据。

关键词：
生物材料；缓释材料；壳聚糖；微球；幽门螺杆菌；体外释放
主题词：
组织工程；生物相容性材料；壳聚糖；疫苗

摘要
背景：包裹幽门螺杆菌全菌蛋白抗原的研究仍处于探索阶段，有关壳聚糖微球包裹幽门螺杆菌全菌蛋白抗原的制备工艺及体外释放性能的文献甚少。
目的：探讨幽门螺杆菌全菌蛋白抗原壳聚糖微球的制备工艺及体外释放特性。
方法：采用沉淀法制备壳聚糖微球，筛选最佳制备工艺及配比、包裹时间，并在电镜下观察微球的形态和粒径。采用壳聚糖微球包裹幽门螺杆菌全菌蛋白抗原，BCA法测定幽门螺杆菌全菌蛋白抗原微球的包裹率、包裹量及体外释放率。
结果与结论：终体积分数为1%的冰醋酸、硫酸钠为交联剂、pH 5.0、滴加交联剂时不粉碎处理为壳聚糖微球最佳制备工艺，电镜观察显示微球表面光滑、形态圆整，具有良好的分散性，多数微球粒径为1.0–5.0 μm。幽门螺杆菌全菌蛋白抗原微球的包裹率为80.4%，包裹量为16.4%，48 h总释放率为19.4%，幽门螺杆菌全菌蛋白抗原微球整体呈缓慢释放状态。结果证实，实验制备的壳聚糖微球对幽门螺样菌全菌蛋白抗原具有良好的包裹率和包裹量，幽门螺杆菌全菌蛋白抗原微球整体呈缓慢释放状态。

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