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Pharmaceutical and immunological evaluation of a single-dose hepatitis B vaccine using PLGA microspheres

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Abstract

The objective of the study was to investigate the feasibility of a single-dose hepatitis B vaccine based on three kinds of poly (D, L)-lactide-co-glicolide acid (PLGA) microspheres. PLGA microspheres loaded with recombinant hepatitis B surface antigen (HBsAg) were formulated using a double emulsion microencapsulation technique. The pharmaceutical characteristics of size, surface morphology, protein loading efficiency, antigen integrity, release of HBsAg-loaded PLGA microspheres and degradation of the polymer in vitro were evaluated. The degradation of the polymer corresponded with the composition of the polymer (lactide/glycolide ratio), molecular weight of the polymer (viscosity) and morphology of the microspheres. These PLGA microspheres were able to continuously release antigen under conditions that mimic the environment in vivo. The single subcutaneous injection of HBsAg-loaded PLGA50/50 microspheres, PLGA75/25 microspheres and a mixture of PLGA50/50, PLGA75/25, and PLGA50/50-COOH microspheres in mice resulted in comparable serum antibody titers to those of three injections of the conventional aluminum adjuvant formulated HBsAg vaccine. Based on these findings in vitro and in vivo, it was concluded that HBsAg was successfully loaded into the PLGA microspheres, which can auto-boost an immune response, and the HBsAg-loaded PLGA microsphere is a promising candidate for the controlled delivery of a vaccine.

Keywords: Hepatitis B surface antigen (HBsAg); Single-dose vaccine; PLGA microspheres; Controlled release; Immunogenicity

1. Introduction

Hepatitis B is one of the most important infectious diseases in the world. Approximately 350 million people worldwide are chronic carriers of the hepatitis B virus (HBV), which accounts for approximately one million deaths annually [1]. In China, there are around 120 million chronic carriers of HBV and 50–80% are expected to develop chronic liver disease, liver cirrhosis and hepatocellular carcinoma [2]. Immunization represents the only known way to prevent the spread of the virus. There are several prominent approaches to the production of recombinant hepatitis B vaccine on a commercial scale [3,4]. Any material that helps antigens or increases the humoral and/or cellular immune response to an antigen is referred to as an adjuvant [5,6], and aluminum adjuvant is the only one approved for human use [5,7]. However, conventional aluminum-adjuvant vaccines require multiple injections at appropriately timed intervals in order to achieve long-lasting and optimal immune responses. The most commonly used immunization schedule for hepatitis B vaccine is three injections given at 0, 1 and 2 months or at 0, 1 and 6 months to provide protective antibody levels. More than 95% of vaccines protect against hepatitis B upon completion of the full three-dose vaccination course [1,2]. But, it is very difficult, especially in developing countries, to maintain a high reimmunization rate in the case of multiple administration immunization programmes. Therefore, as a result of insufficient immunization, infections are still the leading killer among diseases. A major step towards better vaccination coverage

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would be the introduction of single-shot vaccines with the potential to reduce the number of booster administrations [8].

A singe-shot vaccine can be made using microspheres as adjuvants or vehicles [9], from which the encapsulated antigens are released under control over a prolonged period by manipulating the polymer degradation rate, would be of great benefit for developing countries in which health conditions are poor and drop-out rates between the first and the last immunization are high [8,9]. Poly (D, L)-lactide-co-glicolide acid (PLGA) is a primary candidate for use in the preparation of sustained-release vaccines [10–12]. Several reports have demonstrated that PLGA microspheres with peptides, proteins, viruses and other macromolecules provide prolonged immunological or immunotherapeutic responses in animals, thus avoiding the need for multiple injections [13–17]. The microencapsulation may create mechanical, thermal and chemical stresses that affect the antigenicity of protein. In addition, the moisture, acidity, temperature, and un-physiological salt concentration of the microenvironment created during the course of degradation and release, may lead to the destabilization and aggregation for the encapsulated proteins [18]. Some previous papers have shown that HBsAg-loaded PLGA microspheres can elicit an immune response in animals [13,16,17]. There are no extensive studies shown for the relationship between the release of HbsAg and the polymer degradation in those works.

In order to investigate the feasibility of a single-shot of PLGA microspheres with HBsAg, the pharmaceutical and immunological characteristics of HBsAg-loaded PLGA microspheres were evaluated using three different PLGA materials (lactide/glycolide ratios of 50/50, 75/25, and 50/50 with carboxyl ends). The optimization of the immunoresponse was investigated using a mixture of the different kinds of microspheres with different degradation and release rates. It was found that the degradation of the polymers had remarkable influence on the release of vaccine from HBsAg-loaded PLGA microspheres of which the size was less than 10 μm, and the immunological responses induced by the mixture of three kinds of microspheres made from different PLGA materials had accordant effect with that of three injections of the HBsAg-aluminum-vaccine. The HBsAg-loaded PLGA microsphere may be a promising candidate for controlled delivery of Hepatitis B vaccines.

2. Materials and methods

2.1. Materials

Poly (D, L)-lactide-co-glycolide acid (PLGA) with a lactide/glycolide (L/G) ratio of 50/50 (MW 50 kDa, inherent viscosity 0.67 dl/g), PLGA with a L/G ratio of 75/25 (MW 88 kDa, inherent viscosity 0.67 dl/g) and poly (D, L)-lactide-co-glycolide-COOH (PLGA-COOH) with a L/G ratio of 50/50 (MW 18 kDa, inherent viscosity 0.22 dl/g) were purchased from Birmingham Polymers, Inc. (Birmingham, AL, USA). In this paper, the corresponding polymers are referred to as PLGA50/50, PLGA75/25 and PLGA50/50-COOH, respectively. Recombinant Hepatitis B surface antigen (HBsAg, secreted by CHO cells) and Hepatitis B vaccine with aluminum hydroxide as the adjuvant (HBsAg-aluminum-vaccine) were produced under GMP conditions at NCPC Gene Tech Biotechnology Development Co., Ltd. (Shijiazhuang, Hebei, China). Bicinchoninic acid (BCA) protein assay kit (BCA-1 and B9643) was purchased from Sigma Chemicals (St. Louis, MO, USA). Polyvinyl alcohol (PVA 17–88; 88% hydrolyzed) and all other chemicals were of analytical grade and purchased from Beijing Organic Chemicals Plant (Beijing, China).

2.2. Preparation of microspheres loaded with HBsAg

Three kinds of HBsAg-loaded PLGA microspheres (HBsAg-PLGA50/50 microspheres, HBsAg-PLGA75/75 microspheres, and HBsAg-PLGA50/50-COOH microspheres) were prepared using a modified double emulsion method consisting of a 2-step process. Briefly, 100 mg of PLGA was dissolved in 1 ml of methylene chloride and emulsified with 200 μl of a 3.26 mg/ml HBsAg aqueous solution using a probe Ultrasonic Processor (CP130, Cole-Parmer Instrument Co., IL, USA) with an ice bath for 1 min. The resulting primary emulsion (w/o) was dripped into 5 ml of a 5% PVA aqueous solution and homogenized for 3 h at 1000 rpm using a homogenizer at room temperature in order to perform the multiple emulsions (w/o/w) and the residual methylene chloride was evaporated. The resulting microspheres were collected by centrifugation at 10,000 ×*g*, and were washed three times with phosphate-buffered solution (PBS, 12.2 mM Na_2HPO_4, 7.8 mM NaH_2PO_4, 149 mM NaCl) to remove unentrapped HBsAg and PVA, and then were lyophilized for 48 h to obtain a dry powder.

2.3. Particle size and surface morphology

The size of the microspheres was determined using a microscope (OLYMPUS BX50, Japan) in which 200 particles of the microspheres were counted to calculate the average diameter and distribution percentage of the microspheres. The surface morphology was obtained using Environmental Scanning Electron Microscopy (ESEM, FEI Company, Quanta 200 FESEM, USA). The lyophilized microspheres were mounted on aluminum stubs with Carbon Adhesive Discs and imaged at a temperature of 0∼2 °C with a tension of 10–30 kV and a pressure of 3.7–5.1 Torr in a hydrated ESEM mode.

2.4. Assessment of protein loading efficiency

The amount of protein loaded into the HBsAg-PLGA microspheres was determined by dissolving 20 mg of the microspheres in 0.5 ml of a 0.1 M NaOH/5% sodium dodecyl sulfate solution (NaOH/SDS) [12,13,17]. Fifty microliters of supernatants was collected and analyzed for antigen content using a BCA protein assay.

In addition, the HBsAg-PLGA microspheres were dissolved in methylene chloride at 37 °C for 10 min. HBsAg was extracted with 0.3 ml of PBS twice and 0.4 ml of PBS once by gentle mixing and separated by centrifugation at 3000 rpm for 3 min. The aqueous layers from the three extractions were pooled and the amount of protein extracted from the PLGA microspheres was also analyzed by BCA protein assay.

2.5. Protein integrity of HBsAg-loaded PLGA microspheres

In order to confirm the integrity of HBsAg after the microencapsulation process, the integrity of the HBsAg extracted from the PLGA microspheres was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion-high performance liquid chromatography (SEC-HPLC), respectively [17,20,21]. The HBsAg-PLGA microspheres were dissolved in methylene chloride at 37 °C for 10 min. HBsAg was extracted with 0.3 ml of PBS twice and 0.4 ml of PBS once by gentle mixing and separated by centrifugation at 3000 rpm for 3 min. The aqueous layers from the three extractions were pooled and concentrated by Nanocep® (catalog: OD010C33, PALL Corp., USA). For SDS-PAGE analysis, the samples of native HBsAg and concentrated antigen extracted from the HBsAg-PLGA microspheres were loaded onto a 5% stacking gel and subjected to electrophoresis on a 15% separation gel at 200 V (Miniprotean II Electrophoresis Cell, Bio-Rad Lab., CA, USA.). The gel was stained with a 1% (w/v) silver solution. The same samples were also analyzed by SEC-HPLC using a TSK-G5000 PW_{XL} column (Tosoh Corp., Tokyo, Japan) with detection at 280 nm on a TSP-P2000 series HPLC system (Thermo Electron Corporation, MA, USA). The mobile phase was PBS (pH 6.8) delivered at a flow rate of 0.6 ml/min and the injection volume was 100 μl.

In order to confirm protein integrity of HBsAg after the microencapsulation process, the HBsAg after 72 h released from microspheres was analyzed using Western blotting [17]. About 25 mg of HBsAg-PLGA microspheres was placed in an Eppendorf tube containing 1 ml of PBS and shaken with a rotary shaker at 55 rpm and 37 ± 0.5 °C for 72 h. Briefly, the standard and the supernatants in release test (diluted to about 0.1 μg antigen) were blotted onto a wetted nitrocellulose membrane (Hybond-c, 0.45 μm, Amersham Life Science) and blocked at room temperature for 60 min. Then, the membrane was put into the diluted solution of polyclonal anti-HBsAg antibody (purified by NCPC Gene Tech. Biotechnology Development Co., Ltd.) at 37 °C for 60 min. After 3 times of washing, the membrane was moved into the diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Beijing Zhongshan Biotechnological Corp., China) at 37 °C for 60 min. The blots were visualized with 3,3-diaminobenzidine (DAB) solution. Then, the membrane was washed by distilled water to end the reaction.

2.6. In vitro release experiments

The release of HBsAg from the PLGA microspheres was studied as follows. About 25 mg of HBsAg-PLGA microspheres was placed in an Eppendorf tube containing 1 ml of PBS and shaken with a rotary shaker at 55 rpm and 37 ± 0.5 °C. At appropriate intervals, up to 63 days, the samples were centrifuged at 17,800 rpm for 10 min. The supernatants (700 μl) were collected and fresh buffer (700 μl) was added to control the pH, and release experiments were continued. The amount of antigen in the supernatants was determined with the BCA assay. HBsAg release profiles were generated for each microsphere formulation in terms of cumulative antigen release versus time.

2.7. Measurement of the degradation of the polymer

To investigate the degradation of the polymer of microspheres in release experiments, the change in the weight of the microspheres, the pH of the incubation solution, and the molecular weight (MW) of the polymer were measured. Blank PLGA microspheres (about 10 mg) were placed in centrifuge tubes containing 5 ml of PBS and shaken with a rotary shaker at 55 rpm and 37 ± 0.5 °C. At specific times (0, 1, and 2 months), the samples were centrifuged at 10,000 rpm for 10 min. The pH of the supernatants was assayed. The loss of the weight of the microspheres was obtained by weighing the pellet after it was lyophilized. Then the lyophilized microspheres were dissolved in tetrahydrofuran (THF) and the MW of the polymer was determined by gel permeation chromatography (GPC) (Waters 717 plus autosampler, Waters 600 pump, Waters 2414 refractive index detector, Waters Styragel® HR4E column, Waters Corporation, Boston, USA). The detectable MW range was 50–100,000 Da, the column heater was set at 35 °C, and THF was used as the mobile phase at a flow rate of 1 ml/min.

2.8. Immunization protocol and detection of anti-HBsAg antibody

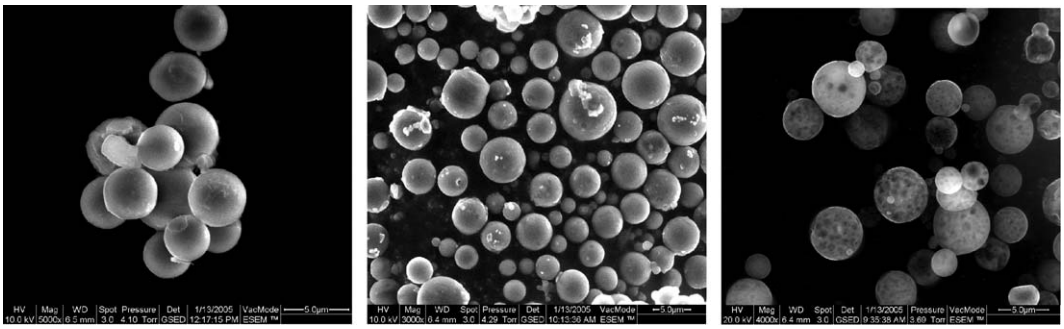
Female BALB/c mice, weighing about 20 g (6–8 weeks old), were obtained from Beijing Weitong Lihua Test Animal Co. (Beijing, China). The mice were maintained on a normal diet throughout the study. To measure anti-HBs antibody (total antibody), six groups of eight mice were injected subcutaneously (sc.) with a quantity of HBsAg, according to the following protocol: (1) three injections of 0.25 ml of HBsAg-aluminum-vaccine (10 μg/ml) at 0, 1 and 2 months, respectively, and a single injection of (2) HBsAg-PLGA50/50-COOH microspheres, (3) HBsAg-PLGA50/50 microspheres, (4) HBsAg-PLGA75/25 microspheres, (5) a mixture of HBsAg-PLGA50/50-COOH, HBsAg-PLGA50/50 and HBsAg-PLGA75/25 microspheres, and (6) 0.25 ml of 0.9% NaCl solution as a blank. The four groups of HBsAg-PLGA microspheres were weighed and dispersed in 0.25 ml of sterile 0.9% NaCl solution and the dose of HBsAg was 7.5 μg/mouse, the same as the total dose for three injections of the HBsAg-aluminum-vaccine. Blood samples were collected from the retro-orbital plexus at 1, 2, 4, 6, 8, 10, 12, 14 and 18 weeks. Sera were separated by centrifugation and stored at −20 °C until assayed.

Anti-HBs antibody was determined by quantitative ELISA using an HBsAb ELISA Kit precoated with purified HBsAg (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., China) as recommended by the manufacturer.

3. Results and discussion

3.1. Size and surface morphology of HBsAg-PLGA microspheres

The HBsAg-PLGA microspheres prepared by the "double emulsion" method in this study were basically spherical with a smooth surface as indicated by morphological examination



A. PLGA50/50 B. PLGA75/25 C. PLGA50/50-COOH

Fig. 1. ESEM micrographs of HBsAg-PLGA microspheres.

using ESEM (Fig. 1). Some of the HBsAg-PLGA microspheres possessed a hollow, donut-like structure and microcaves or little holes on the surface, especially the HBsAg-PLGA50/50-COOH formulation (Fig. 1C). The difference between spherical, donut-like, flattened ball-like and porous-like microspheres may be related to the species of polymer (PLGA) and the hardening and drying process. Because PLGA50/50-COOH has a lower inherent viscosity (0.22 dl/g) than the other two kinds of polymer (0.67 dl/g), the evaporation of methylene chloride during the hardening and drying of PLGA50/50-COOH may be more rapid, leading to forming more microcaves [12,22]. PLGA microspheres with microcave structures were often observed when manufactured using a spray-drying method [16,23].

Various factors including the surface morphology, particle size, polymer composition, viscosity, MW of the polymer, etc. have been shown to affect the release of antigen from microspheres, the degradation of the polymer and the immunogenicity of the antigen loaded in PLGA microspheres [24]. Size was one important factor: the larger the microspheres, the slower the release of vaccine and the longer the immunogenicity [25,26]. Table 1 lists the size determined by optical microscopy and ESEM. Although different methods of measurement of particle sizes showed different values for the three types of PLGA microspheres, the size trend of them was the same as larger order: PLGA50/50-COOH microspheres <PLGA50/50 microspheres <PLGA75/25 microspheres.

3.2. Loading efficiency of HBsAg into PLGA microspheres

The methods most commonly used to determine the protein-loading efficiency into PLGA microspheres are utilizing the BCA protein assay [12] after digestion with NaOH/SDS solution [13,15,19] or extraction with PBS from the dissolution of microspheres by organic solvents (methylene chloride, DMSO, chloroform, etc.) [19]. From the results shown in Table 1, it was concluded that the total amount of antigen encapsulated into microspheres could not be determined accurately by the method of extraction (loading efficiency determined from 18.80% to 27.17%), since a layer of whitish and cloudy protein flocculated between the interface of methylene chloride, DMSO or chloroform and aqueous layers, resulting in an underestimation of the entrapped HBsAg. The protein content of HBsAg aqueous solution obtained from extraction method (85.75%±5.17%) by BCA assay is more close to that of the digestion method (99.59%±0.98%) than that of the protein content of HBsAg-microspheres. This might be due to the protein in aqueous solution being protected by the aqueous layer all the time, and the chance to be immediately in contact with organic solvent is slight, while a portion of protein in microspheres was denatured by organic solvent when microspheres were dissolved by the organic solvent before the PBS being added. Besides, the microspheres could not be completely dissolved and antigen could not be completely extracted from the solvent layer and W/O interface. The results were in accordance with the investigation results of Gupta et al. who showed that up to 70% protein could be obtained after the solvent layer was dried and the polymer was completely digested with 6 M HCl [19]. The digestion method is a relatively accurate technique for the determination of the levels of total protein entrapped in PLGA microspheres [15].

3.3. Integrity of the antigen loaded in microspheres

The retention times of the antigen extracted from microspheres were close to those of the native antigen (13.465 min)

Table 1
Size and loading efficiency of HBsAg in PLGA microspheres

Methods of determination	Size (μm)[a]		Loading efficiency (%)	
	Optical microscopy	ESEM	Digestion (BCA)[b]	Extraction (BCA)[c]
PLGA50/50 microspheres	2.97	4.72	66.50±3.76	18.80±4.89
PLGA75/25 microspheres	4.54	6.39	76.45±4.52	27.17±2.12
PLGA50/50-COOH microspheres	1.72	3.15	73.56±1.92	22.96±5.02

[a] The size was measured by optical microscopy and Environmental Scanning Electron Microscopy (ESEM). The size data were the average of 3–5 measurements.

[b] The loading efficiency was determined by the BCA protein assay after digestion with 0.5 ml of 0.1 M NaOH/5%SDS solutions. The data represent the mean±SD (n=3). The HBsAg-PLGA microspheres were prepared using 0.65 mg HBsAg per 100 mg PLGA polymer by a double emulsion method.

[c] The loading efficiency was determined by the BCA protein assay after extraction with methylene chloride and PBS. The data represent the mean±SD (n=3).

and the purity of the extracted antigen was always above 95% from SEC-HPLC although this method has several limitations; only soluble aggregates can be measured and the size of them is overestimated (data not shown). The antigen's integrity was also examined using SDS-PAGE assays of the antigen extracted from the microspheres. Fig. 2 reveals identical bands for the native and entrapped antigen without any newly distinguishable bands, indicating there was no significant degradation or aggregation of antigens during SDS-PAGE. These results suggest that the integrity of the antigen was not significantly affected by the encapsulation procedure.

Before in vivo immunoactivity investigation, protein integrity of HBsAg after the microencapsulation process was evaluated by Western blots. Fig. 3 reveals identical blots for the native and entrapped antigen without any significant difference, suggesting that protein integrity of HBsAg before and after microencapsulation was unaltered.

3.4. Degradation of PLGA microspheres and release of antigen from the microspheres in vitro

The surface morphology of the HBsAg-PLGA microspheres incubated in PBS at 37±0.5 °C for 40 days was shown in Fig. 4. All the microspheres were in the "erosion phase," especially the PLGA50/50-COOH microspheres that were in a broken-down state. Analysis by GPC showed that the chromatograph curves of the polymer before and after encapsulation were initially fairly broad and symmetrical but gradually developed multiple peaks as the hydrolytic degradation proceeded, with many different and smaller MW components emerging. The changes of MW were summarized in Table 2. The PLGA50/50-COOH microspheres exhibited extensive erosion and breakdown at 1 and 2 months, and the degradation half-life (50% loss of molecular weight, $D_{1/2}$) was 32.7 days. In contrast, the PLGA50/50 microspheres and PLGA75/25 microspheres exhibited incomplete erosion and breakdown at 40 days (Fig. 4), and the $D_{1/2}$ was 41.5 and 116.8 days, respectively. The hydrolysis of PLGA releases lactic and glycolic acids, which may in turn lower the

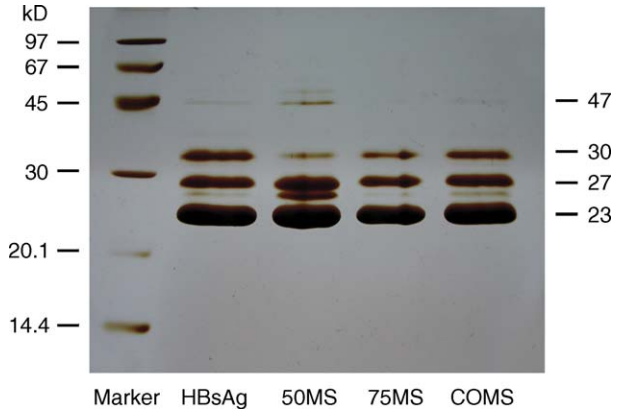


Fig. 2. SDS-PAGE of native HBsAg and entrapped HBsAg extracted from the HBsAg-PLGA50/50 microspheres (50MS), PLGA75/25 microspheres (75MS) and PLGA50/50-COOH microspheres (COMS). The MW in kDa of the peptide brands of marker after gel electrophoretic analysis is shown on the left, and the numbers on the right are those of the peptide brands of antigen.

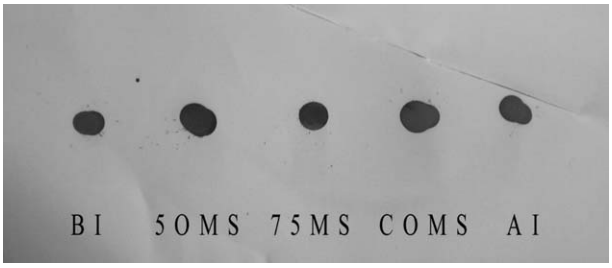


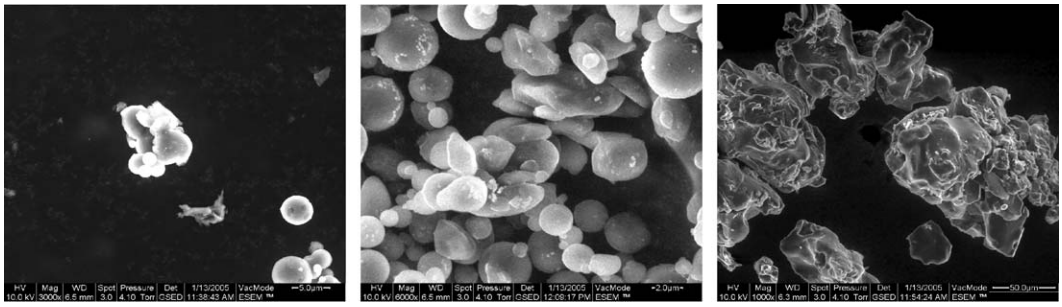
Fig. 3. Western blots of native HBsAg before (BI) and after 3 days incubation in PBS with shaking on a rotary shaker at 55 rpm and 37±0.5 °C (AI), and entrapped HBsAg released from the HBsAg-PLGA50/50 microspheres (50MS), PLGA75/25 microspheres (75MS) and PLGA50/50-COOH microspheres (COMS) incubated in PBS with shaking on a rotary shaker at 55 rpm and 37±0.5 °C after 3 days.

pH of the solution as well as the environment inside of microspheres [11]. The loss of the weight of the microspheres and the changes of pH-accompanied erosion are also given in Table 2. The PLGA50/50-COOH microspheres revealed the largest loss of weight and decrease of pH in the dissolution solution within 2 months, compared with the PLGA50/50 microspheres and PLGA75/25 microspheres. These results corresponded with the $D_{1/2}$ value of a PLGA polymer, which was related to its monomer ratio of L/G [10,27]. The results of our study indicate that in terms of the rate of degradation, the microspheres rank as follows: PLGA50/50-COOH > PLGA50/50 > PLGA75/25.

Profiles of the release of HBsAg from PLGA microspheres based on the three different polymers are shown in Fig. 5. The data are presented as the relative cumulative release of antigen from the microspheres. The PLGA50/50-COOH microspheres released HBsAg faster than the PLGA50/50 microspheres and PLGA75/25 amicrospheres, with respectively, 92.61%±1.37%, 45.2%±5.50%, and 35.35±1.73% of the antigen cumulatively released from microspheres until day-63.

The release of HBsAg seemed to correlate with the morphological changes of microspheres revealed in the ESEM images (Fig. 4), and corresponded to the decrease in MW and weight of microspheres (Table 2), suggesting that the release was controlled mainly by degradation and erosion of the polymer matrix rather than simple diffusion. That is, after microspheres emerge in the aqueous environment, with the water diffusing, the polymer swells and the antigen dissolves. As a consequence of hydrolysis of the ester bonds of polymers and formation of monomers and oligomers, microspheres become irregular in shape, develop craters on the surface, and release protein by diffusion tortuously through the water-filled polymer matrix and/or by escaping from the eroded polymer matrix [10,11,27].

The pores of the PLGA50/50-COOH microspheres might enhance the burst of initial release. When HBsAg-PLGA50/50-COOH microspheres are exposed to an aqueous environment, hydrophilic ends of the polymeric matrix are hydrated by water, the dissolved antigen can be released from the pores by osmotic pressure, and then the carboxylic ends might facilitate the autocatalytic hydrolysis of the ester bonds of the polymer and the erosion of the microspheres [11,22,27]. The PLGA50/50 and PLGA75/25 polymers with relatively high MW and viscosity



A. PLGA50/50 B. PLGA75/25 C. PLGA50/50-COOH

Fig. 4. ESEM micrographs of HBsAg-PLGA microspheres incubated in PBS with shaking on a rotary shaker at 55 rpm and 37±0.5 °C after 40 days.

may form a highly viscous polymer solution in the primary w/o emulsion and this may result in the production of compact and dense microspheres with reduced porosity. Therefore, these microspheres could minimize burst effects and slow down the release of proteins [12,22].

In addition, the size of the microspheres is also an important parameter that influences the rate at which HBsAg is released. As the particles decrease in size, the relatively high surface area per unit volume of the microspheres facilitates contact with the buffer or penetration into the microspheres and at the same time allows a faster diffusion of monomers and oligomers formed as a result of the degradation of the polymer. Then, the acidic (lactic acid and glycolic acid) monomers and oligomers further catalyze the degradation of PLGA microspheres [11,12,22,24,28].

Sánchez et al. [15] reported that PLGA microspheres with a particle size of 20–35 μm released about 50–90% of tetanus toxoid within the first day. Bittner et al. [11] reported that particles of about 18 μm with 9% loading released 60% of bovine serum albumin in an initial burst within the first day. In our study, the particle size was 1–8 μm, the antigen was loaded into microspheres rather than associated with the surface, and the loading percentage was about 0.4% (w/w), so the initial burst release of HBsAg was not observed within 24-h incubation at 37 °C in PBS. It suggested that the larger the particle size, the more antigens located on the surface of the microspheres, and then the more initial burst release. The majority of proteins were released in the first week from HBsAg-PLGA50/50-COOH microspheres, which would afford the initial burst in vivo of the mixture of three different microspheres loaded with HBsAg, corresponding to the priming immunization dose [13]. The release of antigen and the degradation of the microspheres exhibit the same trend, and the sustained release of antigen may induce an extended immunogenic response rather than an initial burst release.

3.5. Immunogenicity

The total antibody induced by the HBsAg-PLGA microspheres in vivo was analyzed by ELISA using plasma taken at different time points from immunized BALB/c mice (Fig. 6). The group that received three injections of 2.5 μg of the HBsAg-aluminum-vaccine at 0, 1 and 2 months is also shown in Fig. 6. The HBsAg-PLGA50/50-COOH microspheres produced rapidly anti-HBs antibody, which continued to fall from 6 weeks onwards compared to the HBsAg-PLGA50/50 and HBsAg-PLGA75/25 microspheres. This finding might be corresponding to the rapid release of HbsAg from PLGA50/50-COOH microspheres (Fig. 5). HBsAg-PLGA50/50-COOH microspheres might have released sufficiently during the 6 weeks and lacked of a persistent antigen stimulation to maintain the antigen concentration in the germinal center of lymphoid tissues in the latter time [29]. It suggested that HBsAg-PLGA50/50-COOH microspheres released sufficiently during the 6 weeks and lacked of an effective booster in the latter time. The fact that HBsAg-PLGA75/25 microspheres released antigen slightly slower than HBsAg-PLGA50/50 microspheres might be due to the higher MW and higher ratio of L/G of the copolymer. The

Table 2
The change of molecular weight determined by GPC, the loss of weight, pH of dissolution solution and 50% loss of MW ($D_{1/2}$) of PLGA blank microspheres incubated in PBS shaking with a rotary shaker at 55 rpm at 37±0.5 °C for 2 months

	Molecular weight of microsphere [a]			Loss of weight of microsphere (%)		pH of dissolution solution [b]		$D_{1/2}$ (day) [c]
Incubation time (month)	0	1	2	1	2	1	2	
PLGA50/50 microspheres	50,378	22,477	3699	7.63	27.69	6.96	4.45	41.5
PLGA75/25 microspheres	86,311	60,202	40,606	2.74	12.41	6.95	6.81	116.8
PLGA50/50-COOH microspheres	16,247	3724	1830	46.84	91.00	4.57	3.56	32.7

[a] The molecular weight (MW) of raw material of PLGA50/50, PLGA75/25 and PLGA50/50-COOH were 50,413, 88,148 and 18,577 Da, respectively, determined by GPC.
[b] pH of the incubation solution was 7.4 in 0 month.
[c] $D_{1/2}$:50% loss of molecular weight was calculated.

mixture of these three microspheres showed an intermediate immune response induced by the rapid release of HBsAg-PLGA50/50-COOH microspheres and the slow release of HBsAg-PLGA50/50 and HBsAg-PLGA75/25 microspheres. Until 3 months post-immunization, the antibody responses for the PLGA50/50 microspheres, PLGA75/25 microspheres and the mixture were comparable to those of the group that received three injections of the HBsAg-aluminum-vaccine ($P>0.05$), and the antibody response for the PLGA50/50-COOH microspheres was significantly lower than that for the group that received three injections of the HBsAg-aluminum-vaccine ($P<0.01$).

Shi et al. [16] reported that HBsAg-loaded PLGA microspheres 25–50 μm in size did not induce a significant immune response after a single injection with a dose of 12 μg of HBsAg, but a single injection of 3 μg of HBsAg-aluminum-vaccine plus 9 μg of HBsAg-PLGA microspheres did induce a response. Priming the mice with the HBsAg-aluminum-vaccine might lead to a greater initial response than immunization with the microspheres alone. The small particles (<10 μm) can easily be phagocytozed and transported by phagocytic APCs into the draining lymph nodes for rapid antigen release, inducing a rapid antibody response, while larger particles (>30 μm) are too large for phagocytosis, so they remain at the injection site and have the effect of continuously stimulating the immune system [26,30]. In our study, the particles were all less than 10 μm, and it is believed that the low molecular weight biodegradable polymer, formulated as small microspheres represents the major priming component of the formulation. In the present study, the antibody level induced by the mixed formulation was slightly greater than that of the HBsAg-PLGA50/50 microspheres and HBsAg-PLGA75/25 microspheres. This might be ascribed to a single injection with the mixture of three HBsAg-loaded microspheres with different particle sizes and degradation and/or release rates. The PLGA50/50-COOH microspheres with a lower MW and faster release properties like an initial priming dose generated a greater total antibody response at an early stage of the immunization, while PLGA50/50 microspheres and PLGA75/25 microspheres with a higher MW and prolonged release properties like booster doses evoked a delayed and sustained response. The results indicated that when a series of microspheres were mixed in vitro and in vivo, the release rate and the immunogenicity of vaccine-loaded PLGA microspheres might be conveniently adjusted to control the fate of the vaccine, achieving an optimal immunogenic effect.

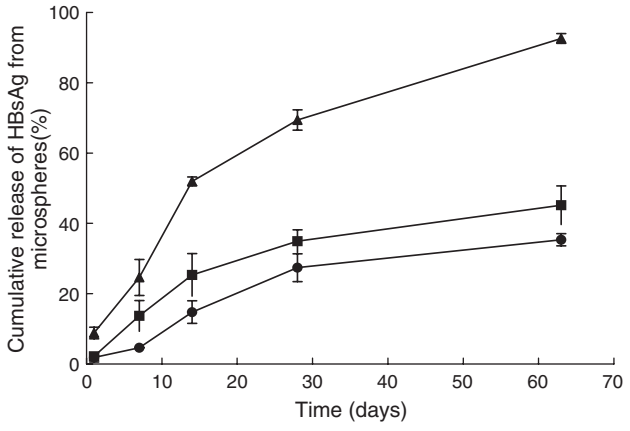


Fig. 5. Relative cumulative release of HBsAg from HBsAg-PLGA50/50-COOH microspheres (▲), HBsAg-PLGA50/50 microspheres (■) and HBsAg-PLGA75/25 microspheres (●) on different days. The HBsAg-PLGA microspheres were incubated in PBS with shaking on a rotary shaker at 55 rpm and 37±0.5 °C.

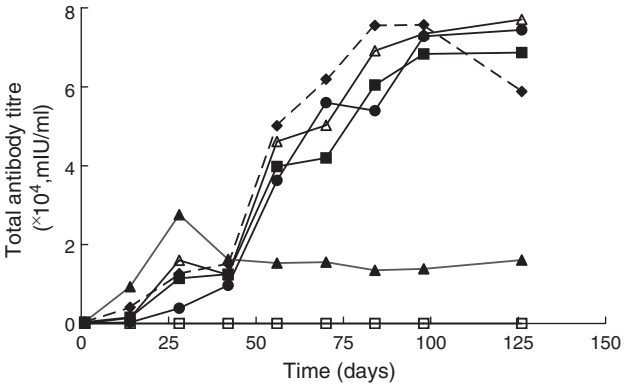


Fig. 6. Geometric mean of total antibody titer ($n=8$) obtained from BALB/c mice after immunizations with three injections of aluminum-HBsAg (2.5 μg) at 0, 1 and 2 months (–◆–), single injection of HBsAg-PLGA50/50 microspheres (–■–), PLGA75/25 microspheres (–●–), PLGA50/50-COOH microspheres (–▲–) and the three above-mentioned mixture (–△–). The blank group given 0.25 ml of 0.9% NaCl solution was expressed as (–□–). The total dose of HBsAg was 7.5 μg/mouse.

4. Conclusion

HBsAg was encapsulated in PLGA microspheres using a double emulsion method. The release of HBsAg from HBsAg-PLGA microspheres was related to the surface morphology of the microspheres, polymer composition (L/G ratio), molecular weight of the polymer (viscosity), etc. A single injection of HBsAg-PLGA microspheres had the capacity to induce a long-lasting immune response in a manner comparable to that of three injections of the HBsAg-aluminum-vaccine; the mixture of microspheres with different release rates in particular induced a strong anti-HBsAg antibody response. The rapid-release PLGA50/50-COOH microspheres were used as a priming dose, and the prolonged-release PLGA50/50 microspheres and PLGA75/25 microspheres were used as an "autobooster dose." Adjustment of the kinetics of antigen release and degradation of polymer could control and manipulate an optimal immune response.

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