Poly-DL-lactide–Poly(ethylene glycol) Microspheres as Oral and Parenteral Delivery Systems for Hepatitis B Surface Antigen

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ABSTRACT: This project was aimed at illustrating the potential use of poly-DL-lactide–poly(ethylene glycol) (PELA) microspheres as a hepatitis B surface antigen (HBsAg) delivery system following subcutaneous (s.c.) or oral immunization over the current injection of an alum-absorbed antigen. The antigen-loading microspheres were elaborated by the solvent-extraction method based on the formation of modified multiple *w*/*o*/*w* emulsion. The microspheres were characterized by their particle size, HBsAg entrapment, and *in vitro* HBsAg release behavior. *Balb*/*c* mice were immunized with an s.c. injection and oral administration of a single dose and two doses of a microsphere formulation. For comparison, the alum-absorbed HBsAg-immunized mice had a following intramuscular (i.m.) injection at weeks 0 and 4. At weeks 8, 10, 14, and 24 postadministration, the blood and saliva samples were collected and detected by the enzyme-linked immunosorbed assay (ELISA) method. A single injection of HBsAg/PELA microspheres could induce a serum lgG antibody level comparable to the two-injection alum-absorbed HBsAg at the 14th week and higher than that at the 24th week. The saliva lgA of peroral groups was significantly higher than that of the s.c. injection of a microsphere formulation and i.m. injection of soluble antigen. These preliminary results demonstrated the potential of oral administration of antigen-loading microspheres in the induction of a secretory immune response, and it is suggested that a single-dose s.c. injection of antigen-loading microspheres would be an efficient immunization schedule to elicit a protective response. © 2002 John Wiley & Sons, Inc. J Appl Polym Sci 83: 850–856, 2002

Key words: biodegradable; drug delivery systems; polyester; microencapsulation; protein

INTRODUCTION

The preparation of protein-loaded microspheres has attracted much attention in recent years. The potential applicability of biodegradable microspheres as drug-, peptide-, protein-, and antigen-delivery systems is based not only on the protection from acidic and proteolytic degradation, but also on the fact that the controlled-release profile could be adjusted by polymer degradation. Especially, sustained-release microspheres using biodegradables such as polylactide (PLA), poly(DL-lactide-*co*-glycolide) (PLGA),[1,2] and poly-DL-lactide–poly(ethylene glycol) (PELA)[3] were investigated and satisfactory results were obtained. Due to the

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existence of a certain amount of hydrophilic poly(ethylene glycol) (PEG) segments in the polymer chains, compared with the commonly used PLA and PLGA, PELA shows much potential in protein-delivery systems. It is beneficial to improve the affinity of the matrix polymer and the protein molecules, which may result in an increase in the protein-entrapment efficiency and a decrease in the burst-release effect to achieve a stable and sustained release profile.[4]

Hepatitis B infection is a significant problem affecting about 5% of the world's population. It is known that 7–10% of acute infection cases become chronic and there is no specific treatment for hepatitis. Hence, immunization represents the only known method to prevent the spread of a virus. The commercially available vaccines for hepatitis B in China are obtained from yeast based on a recombinant DNA technique, whose safety, immunogenicity, and efficiency have been demonstrated by animal trials and clinic studies. But the commonly used immunization schedule is three injections given at the 0, 1st, and 6th month to provide protective antibody levels. The multiple-injection schedules often lead to dropouts among subjects to be immunized, causing a failure of protection. This is also common in developing countries where the population at highest risk lives mainly in isolated rural areas with poor access to health services, the target population is large, and health facilities are unavailable. The development of antigen formulations, which could induce the desired antibody response from a single injection and oral administration, would be of enormous benefit.

Concerning the development of a single-dose vaccine for the hepatitis B surface antigen (HBsAg), previous studies investigated using controlled-release PLGA and PGA microspheres.[5,6] These studies indicated the feasibility of converting the present three-injection schedule for HBsAg into a single-shot therapy. We recently reported the preparation of PELA microspheres as a novel matrix polymer for a hydrophilic drug-delivery system, with entrapped human serum albumin and outer membrane proteins of *Vibrio cholera* and *Leptospira interrogans*. Through investigating factors influencing the particle size and morphology, antigen encapsulation efficiency, and the *in vitro* release profile,[7,8] the resulting PELA microspheres showed effective immunization results by oral and subcutaneous inoculation. In the present study, HBsAg was encapsulated in PELA to prepare antigen-loading microspheres. The *in vitro* antigen-release profile was detected. The serum and saliva antibody responses generated with a single injection or two-dose oral administration of the microspheres were preliminarily evaluated and compared with those observed with a two-dose injection of soluble HBsAg on alum.

EXPERIMENTAL

Materials

PEG (M_w = 6K Daltons) and poly(vinyl alcohol) (PVA, 88% hydrolyzed, M_w = 130K Daltons) were from the Guangzhou Chemical Reagent Department (Guangzhou, China). Copolymer PELA with 11.5% of PEG content was prepared by ring-opening polymerization of DL-lactide and PEG using stannous chloride as an initiator.[9] The actual PEG content of PELA was calculated from the integral height average of hydrogen shown in ^{1}H-NMR (Varian FT-80A, Harbor City, CA). The weight-average molecular weight was 58.9K Daltons with a polydispersity of 2.70, determined by gel permeation chromatography (GPC, Waters, Milford, MA), using polystyrene as a standard. The yeast-derived recombinant HBsAg vaccine was donated by the Chengdu Institute of Biological Products (Chengdu, China) in both an unabsorbed form and an alum-absorbed formation. The unabsorbed form of HBsAg was concentrated by Amicon ultrafiltration before encapsulation.

Preparation of PELA Microspheres Encapsulating HBsAg

HBsAg-encapsulated microspheres were prepared by the emulsion-evaporation technique based on the formation of a modified double emulsion $w_1/o/w_2$ as reported earlier.[7] Briefly, the w_1 phase, containing an aqueous solution of HBsAg, was dispersed into the organic phase (o) consisting of the polymer dissolved in dichloromethane (DCM) and ethyl acetate (EA) (50.0 mg/mL), using a high-speed stirrer for 60 s at room temperature. The primary water-in-oil emulsion (w_1/o) was then immediately added to the external aqueous phase (100 mL of PVA solution) and further emulsified again by a high-speed homogenizer. The organic solvent was extracted by adding 100 mL of 6% isopropanol and the mixture was stirred at a moderate speed at ambient temperature for 3 h. After being centrifuged at 8000 rpm for 8 min

and washed three times with double-distilled water, the microspheres were lyophilized overnight and stored at 4°C in a desiccator.

Characterization of Microspheres

The particle size and its distribution were determined by laser-light diffractometry (Shimazu SALD-2009, Japan). The level of residue of DCM within the microspheres was detected by gas chromatography (GC; Shanghai Analytical Instrument Co., China) and compared with a set of standard samples with a known amount of DCM.

Core loading of HBsAg in the microspheres was determined by extracting the protein from the microspheres and assaying the protein content of the extracted solution. In brief, a known amount of microspheres (ca. 100 mg) in triplicate was dissolved in 500 μL of DCM and extracted three times with 600 μL of double-distilled water. The HBsAg content of the extracted solution was determined using Bradford's method,[10] compared with a standard curve of data obtained by assaying known concentrations of HBsAg solutions. A certain amount of free HBsAg was added to blank PELA microspheres, which was dissolved in DCM, extracted by distilled water, and determined under the same conditions as above. It showed that the extracted solution contained 37.3% fewer proteins than did the original antigen solution. Therefore, the HBsAg entrapped in the microspheres determined by this method was corrected for a 37.3% loss due to inefficient extraction. The amount of encapsulated HBsAg in the microspheres, given as a percentage, indicates the amount (mg) of HBsAg encapsulated per 100 mg of the microspheres.

In Vitro Release Studies

Microspheres (ca. 200 mg) were suspended, in triplicate, in 5.0 mL of pH 7.4 phosphate buffered saline (PBS) and the suspension was incubated at 37°C under continuous orbital rotation at 30 cycles/min (Jiangsu Taichang Medical Apparatus Co., China). At predetermined intervals, the aqueous media were removed with a syringe after centrifugation, while the same amount of fresh PBS was added back to the release medium. The HBsAg content was determined by Braford's method,[10] using a calibration curve obtained with placebo microspheres treated under the same procedure.

Animals

Male *Balb/c* mice aged 8–10 weeks and weighing about 15 g were used for the study and maintained in standard housing and with a standard diet at West China University of Medical Science, Chengdu, China, throughout the course of study.

Immunization

Animals were immunized with the same dose of alum-absorbed antigens or antigens encapsulated in PELA microspheres following oral and subcutaneous (s.c.) routes. Immediately before administration, the required dose of freeze-dried microspheres was weighed and suspended in an appropriate volume of physiological saline (SAL).

Animals were divided into five groups of six mice each. Two groups of mice were starved beforehand, and the antigen-loading microspheres were suspended in 0.2 mL of SAL and fed orally using a blunt-tipped feeding needle inserted into the stomach. The first group (group oral-MS-1) received 10 μg of HBsAg encapsulated in PELA microspheres at 0 and 4th weeks. The other group received two doses of 15 μg of HBsAg at the same time points (group oral-MS-2). Another two groups of mice were inoculated subcutaneously on the left side of the abdomen with a single dose (0.1 mL) of HBsAg/PELA microspheres containing 20 μg of HBsAg (group sc-MS-1) or two doses of microspheres containing10 μg HBsAg at weeks 0 and 4 (group sc-MS-2). The control group (group im-HBsAg) of mice received, through an intramuscular (i.m.) injection, 10 μg of HBsAg on alum at the 0 and 4th weeks.

Mice were bled from the eye sockets at weeks 8, 10, 14, and 24 postadministration. Blood was allowed to clot at 4°C and the serum was harvested in a pasteurized tube. Meanwhile, mice were anesthetized by ethyl ether and saliva samples were collected following an intraperitoneal (i.p.) injection of 0.3 mL of pilocarpine (0.1% w/v). The saliva and serum samples were stored frozen at −40°C until used.

Immunoassay

The specific anti-HBsAg serum lgG and saliva lgA contents were determined by an established ELISA assay.[11] In brief, microtiter plates were coated overnight with 200 μL per well of HBsAg of 100 μg/mL and washed three times at 37°C with 1% bovine serum albumin (BSA) and 0.05% Tween 20 (T20) in PBS 200 μL per well. Serial

dilutions of serum or saliva samples from the study animals in BSA/T20/PBS were added to the wells and incubated at 37°C for 1 h. The plates were washed three times in T20/PBS and 100 μL antimouse lgG (Fc specific, Sigma, St. Louis, MO) or antimouse lgA (two chains, Sigma) diluted in BSA/T20/PBS was added to the wells and incubated at 37°C for 1.5 h. The plates were washed with T20/PBS and avidin–peroxide (Sigma) was added to each well and kept at 37°C for 30 min. *o*-Phenylenediamine (Sigma) was added after washing with another T20/PBS and kept at 37°C. The reaction was stopped after 15 min by the addition of 1*N* H_2SO4 and the plates were detected at 492 nm in an ELISA reader (DG-3022B, Nanjing, China). Samples from individual mice were assayed in triplicate.

Statistical Analysis

The results of a specific antibody were expressed as mean ± standard error for six mice. An unpaired Student's *t*-test was used to compare the means for each study group at the different sample times and to assess the statistical significance. Results were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Characterization of Microspheres

The product is presented as a free-flowing powder, which is easily suspended in SAL for s.c. and oral administration. The SEM spectra of antigen-loading PELA microspheres displayed a smooth spheric surface structure (data not shown). The residual dichloromethane in the microspheres was below 100 ppm, evaluated by gas chromatography, which was lower by far than the limit according to the USP XXII requirement (i.e., 500 ppm for dichloromethane).[12]

The obtained microspheres had a size range from 0.5 to 5 μm with a volume mean diameter of 2.17 μm and standard deviation of 0.178. Also, more than 96% of the population had a diameter less than 5 μm (Fig. 1). More importantly, this is smaller than is the diameter of capillaries, which is suitable for parenteral vaccination due to their efficient prevention of capillary clogging. It is indicated that an intravenously injected particulate substance or drug carriers with an average size below 7 μm are normally taken up by macrophages of the mononuclear phagocytic system, particularly by the Kupffer cells of the liver. Also, it was concluded that with a size of below 10 μm microspheres were taken up into Peyer's patches in the small intestinal mucosa, and then microspheres with a size of below 5 μm were transported to the mesentery lymphoid nodes, liver, and spleen following oral administration.[13] In our previous report, the target distribution of these PELA microspheres in such tissue as liver, spleen, and intestinal mucosa was inspected by SEM, which indicated that the microspheres had successfully reached the immunization-related tissues after injection and oral inoculation.[14]

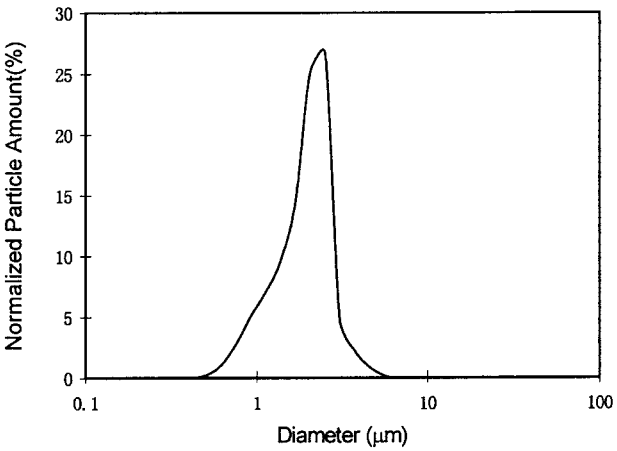

Figure 1 Particle-size distribution, determined by a laser diffraction size analyzer, of PELA microspheres with entrapped HBsAg.

Characterization of HBsAg Encapsulated in PELA Microspheres

The antigens were dispersed within the polymer matrix in a dry state, thus providing an extended shelf life compared with the current formulation of the water solution and obviating the need for a stabilizer or a cold chain. This would be considerably advantageous in developing countries to reduce the overall immunization cost. It was indicated in a previous study that the amount of antigen entrapment influenced the antigen-release behavior from microspheres.[8] The HBsAg entrapment of 1.25% with the loading efficiency of 71.8% was optimized for the present PELA microspheres to achieve a desirable antigen-release profile.

Figure 2 shows the percent release of HBsAg from PELA-10 microspheres against the incubation time. The release pattern was characterized

as a triphasic process, that is, an initial burst release during the first day, gradual release over 1 month, and, lately, the second burst-release phase. The initial burst release of 18.9% of HBsAg was detected during the first incubation day, which was associated with those protein molecules dispersing at or near the microsphere surface diffusing out in the initial incubation time. The gradual release of HBsAg may be due to the swollen inner structure formed by polymer degradation and the contacting of the microsphere matrix with an aqueous release medium and protein diffusion through the swollen phase. The results in Figure 2 show that 48.7% of HBsAg gradually was released from the matrix polymer within 29 days of incubation. As shown in Figure 2, the onset of the second burst release is at day 32 postincubation, and 16.1% of HBsAg diffused out within 4 days. The close correspondence between the onset of the second burst release and the breakdown of the microsphere matrix was investigated in our previous work.[4] When the reduction of the molecular weight of a matrix polymer became significant and the mass loss of microspheres had begun, a critical increase in the porosity and even the breakdown of the matrix was achieved and a higher protein release rate proceeded. The residual 16.0% of HBsAg was detected to slowly release in the next 32 days. This release pattern was thought to mimic the multiinjection of a soluble antigen in the conventional vaccination schedule.

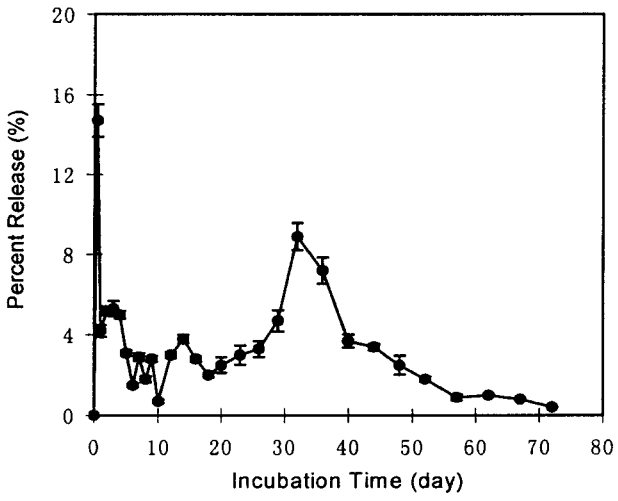

Figure 2 Percent release of HBsAg from PELA microspheres incubated in pH 7.4 PBS at 37°C.

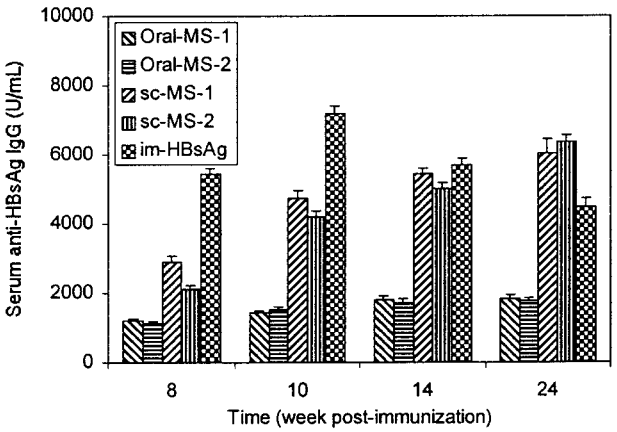

Figure 3 Animal serum anti-HBsAg IgG response at 8, 10, 14, and 24 weeks postimmunization (animals groups were described in the Experimental section; data shown in mean ± standard error for six mice per group).

Serum lgG Response of HBsAg-loading Microspheres

The present study involves one application of a novel matrix polymer PELA to the delivery of HBsAg. The overall goal in our investigation with microencapsulated antigens was to provide a simple, safe, and broadly applicable delivery system with a protective immune response for use in humans. To compare the immune response following s.c. inoculation and oral administration of HBsAg encapsulated in PELA microspheres with the current i.m. injection of HBsAg on alum, mice were vaccinated in separated groups. The serum lgG and saliva lgA responses are summarized in Figures 3 and 4.

As seen from Figure 3, the lgG antibody of alum-adsorbed reached a maximum (7177.9 U/mL) at week 10 and decreased in the following days. The s.c. immunization with a single-dose microsphere formulation induced a significant response (2910.7 U/mL) at week 8, and this was greatly enhanced in the following days. At week 24, the microsphere dose produced an IgG level of 6039.5 U/mL, which is higher than that after administration of soluble HBsAg (4477.1 U/mL). At weeks 8 and 10, according to statistical analysis, the lgG antibody responses to the soluble antigen were statistically greater than were those achieved after s.c. injection of an equivalent amount of antigen encapsulated in the microspheres ($P < 0.05$). But at weeks 14 and 24, there was no significant difference between the lgG response of the single-dose injection of the micro-

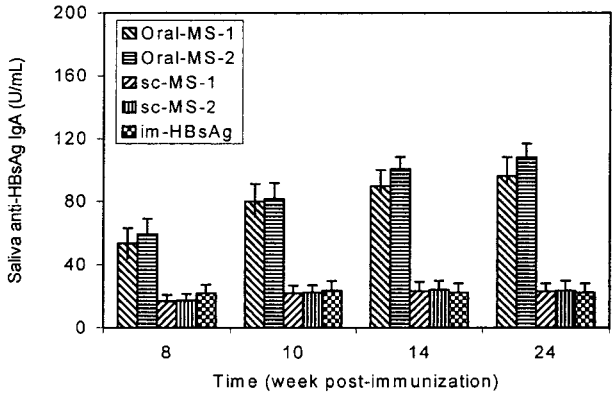

Figure 4 Animal saliva anti-HBsAg IgA response at 8, 10, 14, and 24 weeks postimmunization (animals groups were described in the Experimental section; data shown in mean ± standard error for six mice per group).

sphere formulation and that after administration of the soluble antigen on alum ($P > 0.05$). These levels may be due to the HBsAg-loading microspheres being phagocytized by the macrophages and due to the sustained-release behavior of antigens from the microspheres. Also from Figure 3, at all time points, no significant difference was observed between the immune responses elicited from the two-dose s.c. injection of microspheres containing 10 μg HBsAg and those from a single dose of microspheres containing 20 μg HBsAg ($P > 0.05$). All these preliminary results have a potentially important implication for the development of single-immunization strategies for the induction of a protective immune response.

Singh et al. concluded that a single injection of HBsAg encapsulated in PLGA microspheres could contain an lgG response at a level comparable to the three-injection alum schedule for at least 1 year,[6] but its saliva lgA responses after injection and the serum IgG level after oral administration were not evaluated. It is known that oral administration has many advantages over the routes of administration, such as being safe, convenient, and free of side effects. In the present study, we also examined the elicited lgG response following two-dose oral administration of HBsAg-loading microspheres.

The results in Figure 3 show that the systemic lgG level of 1210.6 U/mL was induced at week 8 after oral administration. Higher lgG responses were detected with increasing time intervals, and 1840.8 U/mL of serum IgG was observed at week 24. This may be due to the uptake of microspheres into the immunization-related tissues after oral inoculation. In our previous study,[14] the antigen-loading PELA microspheres with a size of 1–5 μm had successfully reached such sites as the liver and spleen, the fractured sections of these tissues detected through SEM observation. It was suggested that microspheres were absorbed at Peyer's patches in the small intestine after oral administration, and microspheres of size <5 μm would be expected to be capable of leaving the lymphatic vessels into venous circulation. Such transportation is probably responsible for the ability of peroral microspheres to induce a systemic lgG antibody response to the released antigen.

In previous reports, PLA and PLGA acted as the matrix polymer, and the serum lgG responses evaluated after oral immunization with antigen-loading microspheres were not as strong as were the s.c. administration of microsphere formulations.[15,16] As also shown in Figure 3, at all the time points, the serum lgG levels for two-dose oral administration of HBsAg-loading microspheres were significantly inferior to the i.m. administration of the soluble antigen and s.c. injection of the microsphere formulation ($P < 0.05$). This may be due to the inefficient absorption of microspheres at the intestinal mucosa. Determined by the radio-labeling method in our previous study, only about 8% of the dosed microspheres were absorbed at the intestine site.[14] As seen from Figure 3, serum lgG then showed little difference at all time points ($P > 0.05$) after two-dose oral administration of microspheres containing 30 μg HBsAg, compared with those containing 20 μg HBsAg. From this viewpoint, further development of this carrier system, including the optimization of the matrix polymer and surface properties of the microspheres to improve the uptake efficiency and transportation, is essential to enhance the systemic antibody responses following oral administration.

Saliva lgA Response of HBsAg-loading Microspheres

Figure 4 summarizes the saliva lgA of mice immunized at different schedules. The s.c. immunization with HBsAg-loading PELA microspheres and the two-dose i.m. injection of an equivalent amount of HBsAg on alum showed a similar profile in the saliva lgA level, and no significant difference was observed at every time point ($P > 0.05$). Microspheres after s.c. injection and HB-

sAg released from microspheres may reach the subepithelial tissues or be absorbed by macrophages at mucosa tissues during body circulation and then produce the mucosal immune response.

As seen from Figure 4, the two-dose oral immunization of 30 μg HBsAg in the microsphere formulation elicited slightly higher lgA than that of 20 μg HBsAg. Also, no statistical difference was detected between the two oral groups ($P > 0.05$). But the lgA immune responses of the two oral groups gradually increased up to the 24th week and were significantly higher than was the s.c. administration of the microsphere formulation and i.m. injection of alum-absorbed antigens at every time point ($P < 0.05$). At week 24, oral administration of PELA microspheres at two doses of 20 μg HBsAg produced an anti-HBsAg lgA titer (96.2 U/mL) 4.3 times higher than the current i.m. injection of the same quantity of soluble HBsAg on alum (22.3 U/mL) and 4.2 times higher than a single injection of microspheres (23.2 U/mL). As indicated before,[14] 49.5% of the absorbed microspheres after oral inoculation were located at the intestinal mucosa, which was anticipated to induce the mucosal immune response. These results unequivocally demonstrated the potential of antigen-loading microspheres following oral administration in the induction of an antibody response in the secretory immune system.

As we know, the majority of pathogens enter the host via a mucosal surface and the potentiality of mucosal immunity may offer an effective first line of defense. The enteric delivery may result in induction of a mucosal immune response against pathogens, which usually invade the body through the mucosa. However, the oral administration of antigens was beset with problems, such as the gastrointestinal breakdown by acidic and enzymatic environments. The microspheres with entrapped antigens may protect them from these unfavorable factors and gradually release the entrapped antigens. The elicited secretory lgA response following oral administration of the antigen-loading microspheres should become an efficient immunization strategy against most infectious diseases.

CONCLUSIONS

PELA microspheres with a smooth surface, 2.17 μm of particle size, and 1.25% of HBsAg entrapment were obtained. *In vitro* HBsAg release from PELA microspheres showed a triphasic profile, which mimicked the current immunization schedule of multi-injection. A single injection of HBsAg/PELA microspheres could induce the serum lgG antibody level comparable to the two-injection alum-absorbed HBsAg at the 14th week. The saliva lgA responses of every peroral group were significantly higher than those after s.c. injection of the microsphere formulation and antigen alum absorbed. There was no significant difference in the serum lgG and saliva lgA response for two-dose oral administration of microspheres containing 30 μg HBsAg, compared with those containing 20 μg HBsAg. It is suggested that improving the uptake and transportation efficiency is essential for further development of oral-dosage formulations. These preliminary results also indicated that a single-dose s.c. injection of antigen-loading microspheres would be an efficient immunization schedule to elicit protective responses.

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