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Development of a single-dose stabilized poly(D,L-lactic-co-glycolic acid) microspheres-based vaccine against hepatitis B

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Abstract

The purpose of this study was to develop a stable single-dose vaccine based on recombinant hepatitis B surface antigen (HBsAg) in poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres, in which HBsAg was stabilized by a protein stabilizer (trehalose) and an antacid ($Mg(OH)_2$). The microspheres were prepared by the double emulsion method and characterized by scanning electron microscopy. To neutralize the acids liberated by the biodegradable lactic/glycolic acid based polymer, we co-incorporated into the polymer an antacid, $Mg(OH)_2$, which neutralized the acidity during degradation of the polymer and also prevented HBsAg structural losses and aggregation. The antigen integrity after encapsulation was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by silver staining, isoelectric focusing and Western blotting techniques, which confirmed that antigen remained intact after encapsulation. In-vitro release experiments were performed in phosphate-buffered saline (pH 7.4) and the release of antigen was found to be improved by the protein stabilizer (trehalose). In stability studies, performed at 37°C, the microspheres were found to be stable for 16 days. The immunogenicity of stable microsphere formulations bearing HBsAg was compared with the conventional alum-absorbed HBsAg vaccine in a guinea-pig model. The antibody titre indicated that a single injection of stabilized HBsAg-PLGA microspheres produced a better immune response than two injections of alum-formulated HBsAg vaccine. The findings suggest that recombinant HBsAg can be stabilized by use of a protein stabilizer and antacid during entrapment, and this stabilized preparation can be useful for antigen delivery.

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Introduction

Current paediatric immunization practices involve many injections during the first few months of life because of their poor immunogenicity. Novel delivery methods and alternatives to increase immune responses to proteins have opened up new possibilities for the design of effective single-dose vaccines that may take over from multiple-dose vaccines (Aguado 1993). The development of a single-dose vaccine delivery system based on poly(L-lactic acid) or poly(DL-lactic-co-glycolic acid) (PLGA) microspheres has attracted renewed interest by industry, National Health Institutes and the World Health Organization. The most promising approach is the encapsulation of protein within an injectable microsphere preparation composed of biodegradable and biocompatible polymers such as poly(L-lactic acid) or PLGA, which are approved by the Food and Drug Administration. PLGA microsphere technology has emerged as a promising approach for vaccine formulations, with potential benefits of reducing the number of injections as well as enhancing the immune response (Okada & Toguchi 1995; Igartua et al 1998). Hence, a controlled release vaccine delivery system could overcome many of the disadvantages of multiple-vaccine delivery, such as rate of uptake, cost of administration and inconvenience. Controlled delivery of a desired antigen over a period of 1 to 3 months could be achieved by using PLGA microspheres. In spite of these merits, the applicability of protein delivery using biodegradable polymers is limited, primarily because of protein inactivation during the encapsulation process,

low encapsulation efficiency and instability (Morlock et al 1997; Putney & Burke 1997; Zhu et al 2000; Perez et al 2002).

The integrity of protein structure during encapsulation is not sufficient to develop successful sustained release formulations. Proteins must retain their native active form even after release from the system. Acidity commonly develops in PLGA microspheres because of accumulation of acidic degradation products upon poly-ester hydrolysis, which leads to a decline in pH, and subsequently leads to irreversible inactivation of encapsulated proteins (Mehta et al 1994; Park et al 1995; Brunner et al 1999).

In the present study, PLGA microspheres bearing recombinant hepatitis B surface antigen (HBsAg) were developed. Antigen in the formulation was stabilized by a combination of protein stabilizer (trehalose) and antacid ($Mg(OH)_2$). This delivery system could demonstrate considerable potential for inducing strong immune responses without the requirement for multiple doses. Success in this endeavour would boost efforts by the World Health Organization to achieve high levels of vaccination in developing countries where frequent medical attendance is still a major problem.

Materials and Methods

Materials

PLGA with a lactide/glycolide ratio of 50:50 (MW 40 000–75 000) and polyvinyl alcohol (MW 30 000–70 000) were procured from Sigma Chemical Co. (St Louis, MO, USA). HBsAg (MW ~24 kDa) was obtained from Shantha Biotechnics Ltd (Hyderabad, India). HBsAg used for PLGA entrapment was dialysed and concentrated to 1.2 mg mL^{-1} protein in phosphate-buffered saline (PBS; pH 7.2). Aluminium hydroxide gel (alhydrogel) was purchased from Superfos (Randers, Denmark). All other chemicals and reagents were of analytical grade.

Preparation of HBsAg-PLGA microspheres

PLGA microspheres were prepared aseptically by a double emulsion method at room temperature as reported by Shi et al (2002) with slight modifications. In brief, 800 μL of recombinant HBsAg containing 1.5% w/v trehalose and 2% w/v $Mg(OH)_2$ was suspended in 10 mL of 4% w/v PLGA in methylene chloride and sonicated for 10 s at 50 W in an ice bath (Branson Sonicator 250 New Delhi, India). To this water-in-oil emulsion, 40 mL of 10% w/v aqueous polyvinyl alcohol was added and mixed at high speed with an Ultraturrax T-25 homogenizer (Rose Scientific Ltd, Alberta, Canada) for 10 s to obtain a w/o/w emulsion. The w/o/w multiple emulsion was poured into 50 mL of 0.3% w/v aqueous polyvinyl alcohol with vigorous stirring for 1 h. The microspheres were collected by centrifugation, washed with distilled water, and lyophilized to obtain free-flowing powder. The dried microspheres were stored in a sealed glass vial and placed in a desiccator at 4°C.

Characterization of microspheres

The surface morphology was visualized by scanning electron microscopy (SEM; Jeol 1804, Tokyo, Japan). The samples for SEM were prepared by sprinkling the microsphere powder on a double adhesive tape that stuck to an aluminium stab. The stab was then coated with gold to a thickness of about 300 Å using a sputter coater. The samples were then randomly scanned and photographs were taken.

Estimation of HBsAg content in PLGA microspheres

The loading efficiency of the antigen in biodegradable PLGA microspheres was determined by dissolving 20 mg of the microspheres in 2 mL of 5% w/v sodium dodecyl sulfate (SDS) in 0.1 M sodium hydroxide solution (Singh et al 1997). The amount of antigen was determined by micro bicinchoninic acid assay (Pierce, Rockford, IL, USA) ($n = 6$). Placebo microspheres (without antigen) containing trehalose and $Mg(OH)_2$ were used as control.

In-vitro release of HBsAg from microspheres

HBsAg in-vitro release from PLGA microspheres was carried out in PBS (pH 7.4). Vials containing 50 mg of microspheres and 5 mL of PBS (pH 7.4) were incubated at 37°C on a constant shaking mixer. One vial was withdrawn at various time points (Day 1, 3, 7, 14, 28 and 35). The contents of the vial were centrifuged at 7000 *g* for 10 min and the supernatant containing released HBsAg was collected, estimated by micro bicinchoninic acid assay ($n = 6$) and the same sample was also used to measure in-vitro antigenicity using an enzyme immunoassay (EIA) kit (AUSZYME; Abbott Laboratories, Abbott Park, IL, USA) as described by Shi et al (2002) ($n = 6$). Each sample (HBsAg released from the microspheres) was diluted with 0.2% bovine serum albumin in PBS (pH 7.4) to give three different concentrations, and examined against a linear fitting to the response of control standard samples stored at 4°C. The in-vitro antigenicity of HBsAg was evaluated by the ratio of the EIA response to protein concentration (EIA/protein). Plain HBsAg (sample of internal reference standard, batch no. 62.03, used in Shantha Biotechnics Ltd) was used as a control standard sample at the same concentrations.

To test whether $Mg(OH)_2$ could neutralize the acidic environment, PBS (pH 7.4) medium containing 5 mg polymer microspheres was incubated at 37°C for 2 weeks and the pH was measured. The degradation half-life of PLGA microspheres was also determined by gel permeation chromatography using a Waters 510 pump with a Waters RI-410 refractive index detector (Waters, Bangalore, India). For molecular weight determination, the following conditions were adopted: tetrahydrofuran was the mobile phase at a flow rate of 1 mL min^{-1} and a temperature of 30°C. Incubated polymer microspheres were dissolved in tetrahydrofuran (0.25% w/v polymer sample in tetrahydrofuran), filtered and then injected (20 μL) into a set of four μ-Styragel columns (Waters) with nominal pore sizes of 10^5, 10^4, 10^3

and 100 Å. Average molecular weights were calculated using a series of polystyrene standards as described by Barrera et al (1995) (n = 6).

Percentage aggregation was determined as described by Zhu et al (2000). Briefly, incubated polymers were removed from release medium, dried and dissolved in acetone. After centrifugation and removal of the polymer solution, the remaining HBsAg pellet was reconstituted in PBS (pH 7.4) containing 0.2% Tween-20 (PBST) and incubated (37°C) overnight before determining the protein content. This gave a measure of the encapsulated water-soluble protein. Any aggregates were collected by centrifugation and incubated (37°C for 30 min) in denaturing solvent (PBST, 6 M urea, 1 mM EDTA). Analysis of protein concentration gave the amount of non-covalently bonded HBsAg aggregates. The same procedure was repeated with reducing solvent (10 mM dithiothreitol in denaturing solvent) to determine the amount of disulfide-bound aggregates (n = 6).

Determination of the structural integrity of HBsAg

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to examine the integrity of the antigen (Laemmli 1970; Singh et al 1997). HBsAg was extracted by dissolving the microspheres in 2 mL of 5% w/v SDS in 0.1 M sodium hydroxide solution (Singh et al 1997). The extracted antigen was concentrated and loaded onto a 3.5% stacking gel and subjected to electrophoresis on a 12% separation gel at 200 V (Bio-Rad, Hercules, CA) until the dye band reached the gel bottom. The gel was then stained with silver staining solution and developed using formaldehyde and citric acid solution. To examine potential structural alterations of encapsulated HBsAg, isoelectric focusing (IEF) was also performed to characterize the encapsulated antigen (Zhu et al 2000).

Stability studies

The stability of HBsAg within the PLGA microspheres core environment was investigated. Lyophilized HBsAg-PLGA microspheres stabilized with 1.5% w/v trehalose were incubated at 37°C (n = 6) (Shi et al 2002). One vial was withdrawn at each time point (Day 1, 4, 7, 10, 13, 16 and 19). The antigen was then extracted (Singh et al 1997) and examined using SDS-PAGE analysis (Laemmli 1970) followed by blotting the gels (Western blot) onto a cellulose nitrate membrane in glycine/Tris transfer buffer at 10 V for 1 h (Bio-Rad). The membrane was blocked for 1 h in 5% w/v skimmed milk powder in PBST and incubated for 1 h with polyclonal rabbit anti-HBsAg. After three washings with PBST, the blot was incubated for a further 1 h with goat anti-rabbit IgG conjugated to enzyme. Three washings of PBST were given at an interval of 15 min and the bands were visualized. The in-vitro immunogenicity was also measured by EIA/protein ratio determination. Alum-adsorbed HBsAg incubated at 37°C was used as a control. All EIA/protein ratio values are the average of six measurements (n = 6).

In-vivo studies

Guinea-pigs (350–400 g) were used for in-vivo studies. Animals were housed in groups of six (n = 10) with free access to food and water. They were deprived of food 3 h before subcutaneous immunization. The Institutional Animals Ethical Committee of Dr Hari Singh Gour University approved the study. The studies were carried out according to the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. The formulations were prepared by dispersing the microspheres in sterile normal saline containing a 1% solution of sodium carboxy methylcellulose to achieve a single dose of 20 $\mu g\,mL^{-1}$ (Jameela et al 1994); a volume of 1 mL was injected into the back of the guinea-pigs. The control group received a 10-μg dose of alum-adsorbed HBsAg and a booster was given after 4 weeks of primary immunization. At 2 weeks after administration of the booster, the animals were bled by cardiac puncture at 0, 15, 30, 45 and 60 days. The concentration of anti-HBsAg antibody in the collected blood was determined using the solid-phase enzyme-linked immunoassay (AUSAB; Abbott Laboratories) (n = 6) (Nellore et al 1992). To signify actual antibody concentration (antibody titre) in $mIU\,mL^{-1}$, a standard curve was prepared using the calibrated anti-hepatitis B panel provided by Abbott Laboratories. Antibody response was plotted as log of anti-HBsAg antibody titres ($mIU\,mL^{-1}$) versus time in days.

Statistical analysis

The effect of increasing concentrations of $Mg(OH)_2$ (0.5, 1.0, 1.5 and 2.0% w/v) on percent aggregation, PLGA degradation and pH of the medium of each formulation was analysed by one-way analysis of variance (GraphPad; InStat, San Diego, CA) followed by post-hoc Tukey's multiple comparison test (n = 6). The effects of increasing concentrations of trehalose (0.5, 1.0, 1.5 and 2.0% w/v) and time on percent cumulative release of HBsAg, and in-vitro antigenicity of the each PLGA formulation, were evaluated by one-way analysis of variance and post-hoc comparisons of the means of individual formulations were done by using Tukey's multiple comparison test (n = 6). One-way analysis of variance followed by post-hoc Tukey's test was also used to measure the in-vitro antigenicity during stability studies. The effect of different formulations of PLGA and time on the antibody response of each formulation was analysed by one-way analysis of variance followed by post-hoc Tukey's multiple comparison test (n = 6). Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Physical characterization of PLGA microspheres containing HBsAg

The PLGA microspheres prepared by the double emulsion method were spherical in shape with a smooth surface. The

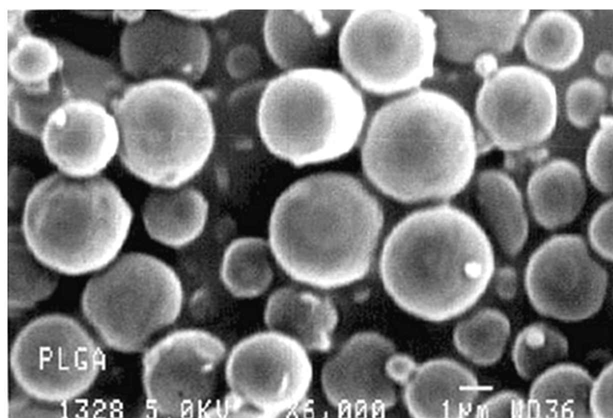

Figure 1 Scanning electron microscopy photograph of poly(D,L-lactic-co-glycolic acid) microspheres encapsulating recombinant hepatitis B surface antigen.

Table 1 Neutralization effect of $Mg(OH)_2$ on the behaviour of poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres encapsulating recombinant hepatitis B surface antigen (HBsAg)

Property	Concentration of $Mg(OH)_2$ (w/v)				
	0%	0.5%	1.0%	1.5%	2.0%
Aggregation (%)[a]	61 ± 6	48 ± 3	32 ± 4	12 ± 2	1.5 ± 0.5*
PLGA degradation t½ (days)[b]	12	16	21	25	30*
pH of the medium[c]	3.1	4.2	5.5	6.4	7.0*

[a]HBsAg was extracted from microspheres (5 mg) after incubation in phosphate-buffered saline (PBS; pH 7.4) at 37°C for 2 weeks (mean ± s.e.m., n = 6). [b]t½ is the time when the PLGA Mr was reduced to half of the original Mr (determined by gel permeation chromatography) during incubation in PBS at 37°C (n = 6). [c]PBS medium containing 5 mg polymer microspheres after incubation at 37°C for 2 weeks (n = 6). 0.5, 1.0 and 1.5% $Mg(OH)_2$ showed non-significant differences from control (without $Mg(OH)_2$), $P > 0.05$. *2.0% $Mg(OH)_2$ showed a significant difference from control, $P < 0.05$.

surface of these microspheres, as observed by SEM, was free from any pores or cracks (Figure 1). The SEM results suggested that the particle size of individual microspheres was over the range 1–10 μm. Microspheres less than 10 μm in size are suitable for maximum adjuvant effect: they are capable of entering into lymph nodes and produce high concentration gradients of antigen over an extended time period. The lymph node accumulation promotes the interaction of encapsulated antigen with antigen presenting cells such as macrophages and dendritic cells (Eldridge et al 1991; O'Hagan et al 1993; Ryan et al 2001). The loading efficiency of HBsAg-PLGA microspheres was over the range 80–85%, as determined by the micro bicinchoninic acid assay. Microspheres were earlier standardized using a model antigen, ovalbumin, to optimize process parameters to obtain uniform and reproducible batches of microspheres.

Because the actual size of individual microspheres was over the range 1–10 μm, the preparation could not be sterilized by filtration. Terminal sterilization of the microspheres (UV irradiation) also led to degradation of the antigen. Microspheres were therefore prepared aseptically to ensure that the final microspheres were sterile and to protect the integrity and activity of HBsAg.

Hydrolysis of PLGA causes the release of lactic and glycolic acids, which induce acidic pH (< 3) inside the microenvironment of the microspheres. This drop in pH is the potential cause of irreversible inactivation of encapsulated proteins (Heller 1990; Zhu et al 2000; Perez et al 2002). In order to prevent the drop in pH, $Mg(OH)_2$ was incorporated at different concentrations (0.5, 1.0, 1.5 and 2.0% w/v) into the microspheres to prevent structural losses and protein aggregation. To confirm the neutralization of the acidic environment by $Mg(OH)_2$, the percentage aggregation, PLGA degradation and pH of the release medium were examined (Table 1). The effects of increasing concentrations of $Mg(OH)_2$ (0.5, 1.0, 1.5 and 2.0% w/v) on percentage aggregation, PLGA degradation and pH of the medium of each formulation were measured by one-way analysis of variance followed by post-hoc Tukey's multiple comparison test. The higher concentrations of $Mg(OH)_2$ (2.0%) showed significant differences ($P < 0.05$) in percentage aggregation, PLGA degradation and pH of the medium compared with the formulation without $Mg(OH)_2$. As predicted, as more $Mg(OH)_2$ (2.0%) was incorporated into the PLGA microspheres, the percentage aggregation was reduced from 61% to 1.5%, the degradation half-time was extended from 12 days to 30 days, and the pH of the release medium dropped from 7.4 to 7.0 (pH 3.1 was found without $Mg(OH)_2$) (Table 1). SDS-PAGE analysis showed that a large degree of fragmentation occurred in the absence of $Mg(OH)_2$ (data not shown).

The preparation of PLGA microspheres requires the use of discordant solvents that may degrade proteins. When the microspheres are exposed to physiological challenges (temperature, pH and osmotic strength), destabilization of the protein molecules can occur (Aguado and Lambert 1992; Esparza & Kissel 1992; Alonso et al 1993, 1994; Crotts & Park 1998; Perez & Griebenow 2001). It was necessary to stabilize the protein during both the encapsulation process and the release of protein from the microspheres in-vivo. The microspheres were therefore co-encapsulated with the potential protein stabilizer, trehalose. It was also hypothesized that the protein stabilizer could shield the antigens from the organic solvent via preferential hydration of their surface, thus preventing protein–interface exposure to deleterious solvent effects (Chang & Gupta 1996; Cleland & Jones 1996; Perez & Griebenow 2001).

In-vitro release of HBsAg

The HBsAg release pattern from PLGA microspheres with trehalose at different concentrations (0.5, 1.0, 1.5 and 2.0% w/v) was compared with PLGA microspheres without trehalose. Statistical analysis of the effect of increasing

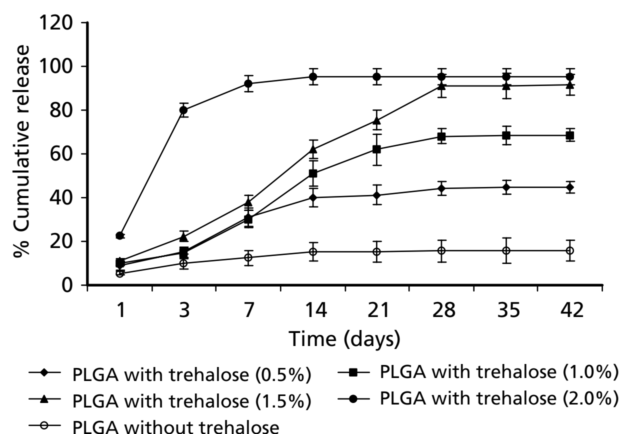

Figure 2 In-vitro cumulative release of recombinant hepatitis B surface antigen stabilized with 0.5, 1.0, 1.5 and 2.0% trehalose from poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres (n = 6). 1.5 and 2.0% trehalose showed significant differences compared with control (PLGA microspheres without trehalose). $P < 0.001$.

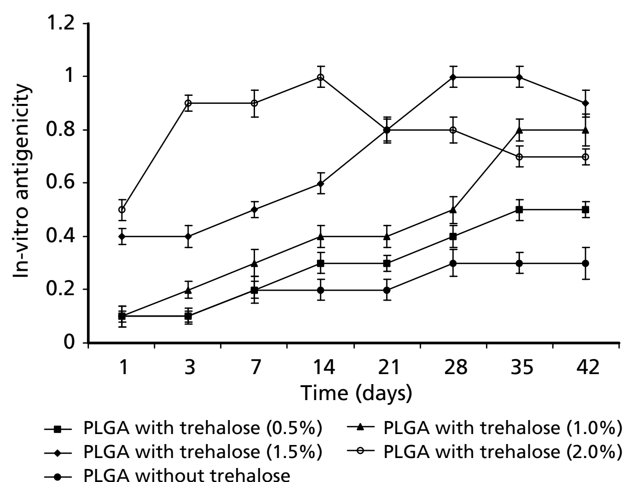

Figure 3 In-vitro antigenicity (ratio of the enzyme immunoassay response to protein concentration) of hepatitis B antigen stabilized with 0.5, 1.0, 1.5 and 2.0% trehalose encapsulated in poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres during in-vitro release studies (n = 6). 1.5 and 2.0% trehalose showed significant differences compared with control (PLGA microspheres without trehalose). $P < 0.001$.

concentrations of trehalose on HBsAg release indicated that 1.5% and 2.0% w/v trehalose showed a significant ($P < 0.001$) increase in the release of HBsAg when compared with PLGA microspheres without trehalose. PLGA microspheres without trehalose released only $15.2 \pm 5.0\%$ of the loaded HBsAg on Day 14 and no further release was observed up to 42 days (Figure 2). This could be owing to antigen inactivation or aggregation at the w/o interface (first emulsion step) (Perez et al 2002). The best approach to prevent HBsAg denaturation and aggregation at the interface is the addition of a protein stabilizer (trehalose) to shield the HBsAg from the organic solvent (Cleland & Jones 1996). Since the protein stabilizer (trehalose) reduced denaturation at the w/o interface, the payload of HBsAg was increased and this was reflected in augmented cumulative percent release. Moreover, sugars (e.g. trehalose, sucrose) have appreciable solubility in aqueous media. They dissolve rapidly from the matrix leaving a porous matrix, which in turn releases antigen/bioactive relatively faster.

As the trehalose concentration increased from 0.5% to 2.0% w/v during entrapment, release of HBsAg was also increased up to > 90%. However, a higher concentration of trehalose (2.0% w/v) increased the initial burst of antigen: $80 \pm 2.8\%$ of HBsAg was released within 3 days. Microspheres stabilized with 1.5% trehalose increased the release of HBsAg slowly up to 42 days as compared with the higher concentration (2.0%) (Figure 2). Therefore, we chose 1.5% trehalose for further studies to provide a more stable environment for HBsAg during encapsulation and during its subsequent release from the system. In our previous experiments, other amphipathic excipients (protein stabilizers), including bovine serum albumin, mannitol and sucrose, were also co-encapsulated, but only trehalose was useful in maintaining protein integrity and also better release of the antigen from the microsphere formulations (results not shown).

PLGA microspheres with trehalose (0.5, 1.0, 1.5 and 2.0% w/v) and PLGA microspheres without trehalose were examined for in-vitro antigenicity during in-vitro release studies. The in-vitro antigenicity of HBsAg was evaluated by the EIA/protein ratio. PLGA microspheres with trehalose at 0.5, 1.0, 1.5% w/v showed an EIA/protein ratio value of 0.5 ± 0.06, 0.8 ± 0.06 and 0.9 ± 0.04, respectively, on Day 42 (Figure 3). PLGA microspheres with trehalose at 2.0% exhibited an EIA/protein ratio value of 0.9 ± 0.04 on Day 3, which dropped to 0.7 ± 0.05 from 35 days (Figure 3). The effect of trehalose concentration and time on in-vitro antigenicity of each formulation was statistically analysed and a significant ($P < 0.001$) increase in in-vitro antigenicity was found at a trehalose concentration of 1.5% and 2.0% when compared with PLGA microspheres without trehalose. This drop could be owing to denaturation of the antigen as the released antigen was continuously exposed to in-vitro release medium at 37°C from Day 3 to Day 42. This finding suggested that 1.5% trehalose provided better controlled release and also maintained the in-vitro antigenicity up to 42 days. PLGA microspheres without trehalose exhibited poor in-vitro antigenicity of HBsAg (0.3 ± 0.03). Thus, stabilization of HBsAg using 1.5% trehalose is a feasible and attractive approach for the formulation of injectable microspheres.

Confirmation of the structural integrity of the antigen

Structural integrity of the HBsAg was confirmed by SDS-PAGE and IEF techniques. SDS-PAGE analysis followed by

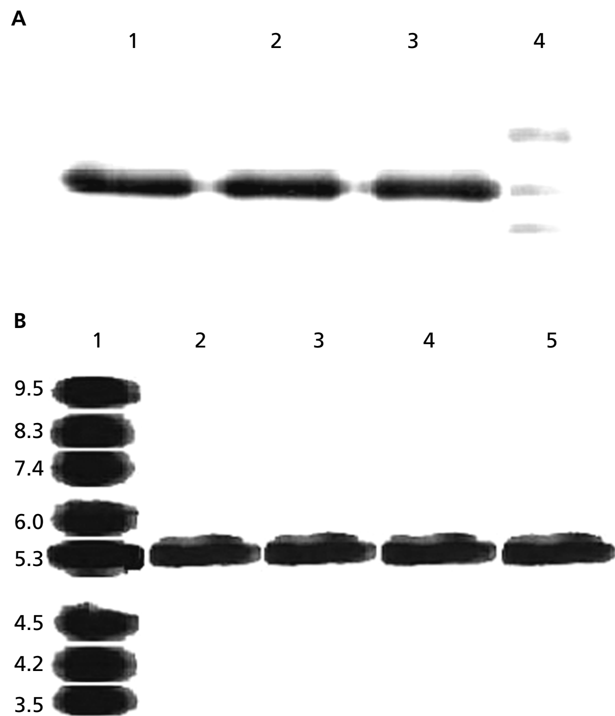

Figure 4 A. SDS-PAGE analysis of recombinant hepatitis B surface antigen (HBsAg) encapsulated in poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres. Lane 1: unencapsulated HBsAg; lanes 2 and 3: encapsulated HBsAg with trehalose; lane 4: encapsulated HBsAg without trehalose. B. Isoelectric focusing analysis of recombinant HBsAg encapsulated in PLGA microspheres. Lane 1: standard markers; lane 2: unencapsulated HBsAg; lane 3: alum-adsorbed HBsAg; lanes 4 and 5: encapsulated HBsAg with trehalose.

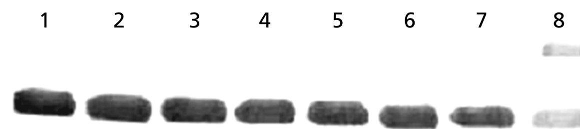

Figure 5 Western blot analysis showing immunoreactivity of recombinant hepatitis B surface antigen (HBsAg) during stability studies at 37°C. Lane 1: unencapsulated HBsAg; lane 2: Day 1; lane 3: Day 4; lane 4: Day 7; lane 5: Day 10; lane 6: Day 13; lane 7: Day 16; lane 8: Day 19.

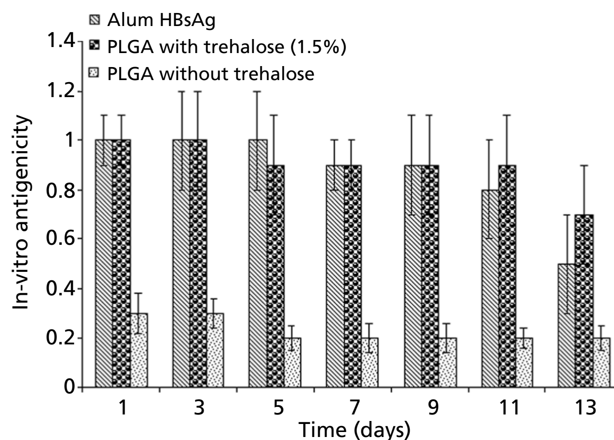

Figure 6 In-vitro antigenicity (ratio of the enzyme immunoassay response to protein concentration) of recombinant hepatitis B surface antigen (HBsAg) during stability studies at 37°C (n = 6) ($P < 0.001$). Alum HBsAg, alum-adsorbed HBsAg; PLGA, poly(D,L-lactic-co-glycolic acid. PLGA with trehalose (1.5%) and alum-adsorbed HBsAg showed significant differences from control (PLGA without trehalose), $P < 0.001$.

silver staining (Figure 4A) revealed identical bands for the native and entrapped antigen with protein stabilizer (trehalose). However, the integrity of the HBsAg without the protein stabilizer was altered after microencapsulation, possibly owing to antigen unfolding or aggregation at the o/w interface. These results provide convincing evidence that the protein stabilizer (trehalose) has a positive role in preventing the inactivation of antigen during the encapsulation process.

IEF analysis is one of the best techniques to test protein degradation (i.e. deamidation of the encapsulated and released antigen) (Zhu et al 2000), so the potential structural alteration of stabilized HBsAg was further confirmed by IEF analysis (Figure 4B). Results suggested that the isoelectric point of HBsAg (IEF 5.2–5.5) remained the same for the native, alum-adsorbed HBsAg and encapsulated HBsAg together with stabilizer.

Stability studies

It was important to confirm the stability and activity of proteins during their storage and release from microspheres. Stability studies were performed at 37°C. The confirmation and activity of HBsAg was examined by Western blot analysis. The encapsulated antigen was extracted from the PLGA microspheres and concentrated using amicon ultrafiltration before being used for the Western blot. The immunoreactivity of encapsulated HBsAg was unaltered up to 16 days as observed with Western blot analysis (Figure 5). In-vitro immunogenicity of encapsulated HBsAg was evaluated by EIA kit and the EIA/protein ratio was found to be 0.9 ± 0.2 up to 16 days (Figure 6). One-way analysis of variance followed by post-hoc Tukey's test was performed to measure the in-vitro antigenicity during stability studies. The 1.5% w/v trehalose concentration and alum-adsorbed vaccines showed a significant ($P < 0.001$) increase in in-vitro antigenicity when compared with PLGA microspheres without trehalose. Alum-adsorbed HBsAg incubated at 37°C was used as a control and gave an EIA/protein ratio value of 0.9 ± 0.3 up to 16 days. After Day 16, the EIA/protein value of control and stabilized HBsAg-PLGA microspheres dropped to 0.5 ± 0.2 and 0.7 ± 0.2, respectively. Hence, the in-vitro antigenicity of alum-adsorbed HBsAg (control) and HBsAg-PLGA microspheres stabilized with 1.5% trehalose and 2% $Mg(OH)_2$ was found to be equal for 16 days. Thus, HBsAg was successfully encapsulated and remained stable at 37°C for 16 days. The inherent stability of unencapsulated HBsAg at a range of acidic pH values

Table 2 In-vitro antigenicity of recombinant hepatitis B surface antigen (HBsAg) formulations at different pH values during inherent stability studies at 37°C

HBsAg formulation	pH 3	pH 4	pH 5	pH 6	pH 7
HBsAg in solution at 37°C for 1 week	0.5	0.7	1.0*	1.1*	1.0*
HBsAg in PLGA at 37°C for 1 week	0.9	1.0	1.0	1.0	1.0
HBsAg in solution at 4°C (no 37°C incubation)	1.0	1.0	1.0	1.0	1.0

PLGA, poly(D,L-lactic-co-glycolic acid). Antigenicity was evaluated by the ratio of the enzyme immunoassay response to protein concentration (EIA/protein). EIA/protein ratio values are the average of six measurements. pH 3 and pH 4 showed non-significant differences from control (PLGA encapsulated without $Mg(OH)_2$ at 37°C), $P > 0.05$. *pH 5–7 showed significant differences from control (PLGA encapsulated without $Mg(OH)_2$ at 37°C), $P < 0.05$. (Unencapsulated plain hepatitis B at 4°C was used as positive control).

(pH 3–7) was also studied at 37°C for 1 week. The results indicated that HBsAg lost ~30–50% of in-vitro antigenicity (EIA/protein ratio), when the pH was decreased to below 5.0, and remained ~100% active over the range of pH 5–7 (Table 2). HBsAg in PLGA microspheres gave an EIA/protein ratio value of 1.0 ± 0.1 (~100% active) over the range of pH 3–7 (n = 6). The effects of pH 3–7 on in-vitro antigenicity of each formulation was analysed and a significant ($P < 0.05$) increase in in-vitro antigenicity was found over the range of pH 5–7 when compared with pH < 5. Unencapsulated HBsAg stored at 4°C (no 37°C incubation) was used as a control. As pH inside PLGA microspheres is believed to be below 5 (Table 1), $Mg(OH)_2$ was co-incorporated into the PLGA microspheres to neutralize the acidity during degradation of the polymer and maintain the pH at between 5 and 7, at which the antigen was more stable (~100 active) ($P < 0.05$).

In-vivo studies

The immunogenicity of the HBsAg-PLGA microspheres was compared with the conventional alum-adsorbed HBsAg vaccine in guinea-pigs. PLGA microsphere-based formulation with trehalose (single injection), PLGA microsphere-based formulation without trehalose (single injection), alum-adsorbed HBsAg vaccine (single injection) and alum-adsorbed HBsAg vaccine (single and booster injections) were compared. The effect of different formulations of PLGA and time on the antibody response of each formulation was analysed by using one-way analysis of variance followed by post-hoc Tukey's multiple comparison tests. Alum-adsorbed HBsAg and PLGA microspheres with trehalose and $Mg(OH)_2$ showed a significant ($P < 0.05$) increase in the level of anti-HBsAg antibodies. Day 60 (i.e. 90 days after the primary dose) serum antibody titres indicated that a single injection of the HBsAg-PLGA formulated vaccine with trehalose produced an almost equivalent immune response when compared with two injections (with booster) of

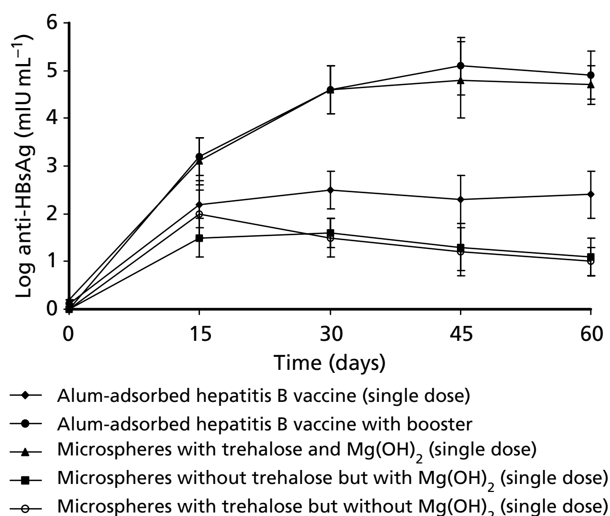

Figure 7 Anti-recombinant hepatitis B surface antigen (HBsAg) antibody response of different HBsAg formulations (n = 6). Alum adsorbed hepatitis B vaccine (booster dose) and microspheres with trehalose and $Mg(OH)_2$ (single dose) showed significant differences from control (microspheres without trehalose and $Mg(OH)_2$), $P < 0.05$.

alum-adsorbed HBsAg vaccine ($P < 0.05$) (Figure 7). The HBsAg-PLGA formulated vaccine without trehalose and a single injection of alum-adsorbed HBsAg vaccine did not result in any significant antibody titres. Shi et al (2002) and Singh et al (1997) reported that a single injection of HBsAg-PLGA formulated vaccine did not result in any significant antibody levels, however, a single injection of a mixture of alum- and PLGA-formulated HBsAg resulted in a good antibody response that mimicked multiple injections of alum-adsorbed HBsAg vaccine. In contrast, in the present study, significant antibody titres were found when a single injection of stabilized HBsAg-PLGA microspheres was administered ($P < 0.05$). If the released HBsAg from the microspheres with stabilizer was not antigenically active, the antibody titre could have been similar to the antibody titre produced by the single dose of the HBsAg-PLGA microspheres without stabilizer or less than the antibody titre produced by the single dose of the alum-adsorbed HBsAg vaccine. Thus, it may be concluded that the HBsAg released from the stabilized PLGA microspheres was active enough to match the two injections of the alum-adsorbed HBsAg vaccine. $Mg(OH)_2$ could also have acted as an additional adjuvant, similar to the well known adjuvant $Al(OH)_3$. Therefore, an additional formulation (HBsAg in PLGA with trehalose but without $Mg(OH)_2$) was made. The in-vivo evaluation of this system did not lead to significantly higher and more prolonged antibody levels (up to 60 days) than those measured for the PLGA microspheres without trehalose but with $Mg(OH)_2$ ($P > 0.05$) (Figure 7). This finding indicates that $Mg(OH)_2$ had no additional adjuvant effect and was only used to prevent the pH drop within PLGA microspheres (Table 1). Thus, trehalose provided a stable environment for HBsAg during entrapment and subsequent release from the system.

Conclusion

The aim of the present study was to design an approach for stabilizing HBsAg in PLGA microspheres. The polymer selected for controlled drug delivery was biodegradable, safe (tissue compatible), permeable, easy to process and stable in-vivo. By adding a protein stabilizer (trehalose) and an antacid ($Mg(OH)_2$), the stability of the antigen was substantially enhanced during entrapment and release from the microspheres. The protein stabilizer played a key role in preventing the inactivation of antigen during the encapsulation process and subsequent release. SDS-PAGE and IEF confirmed that antigen remained intact after encapsulation. In-vivo immunogenicity studies of the formulations in guinea-pigs showed that a single injection of PLGA microspheres resulted in good anti-HBsAg antibody levels, which mimicked booster injections of alum-adsorbed HBsAg vaccine.

The results indicate that PLGA microspheres encapsulating stabilized HBsAg have potential application in the field of vaccine delivery and could be an appropriate choice for the development of a single-dose vaccine against hepatitis B.

References

Aguado, M. T. (1993) Future approaches to vaccine development: single-dose vaccines using controlled release delivery systems. *Vaccine* **11**: 596–597

Aguado, M., Lambert, P. (1992) Controlled release vaccines: biodegradable polylactide/polyglycolide (PL/PG) microspheres as antigen vehicles. *Immunology* **184**: 113–125

Alonso, M. J., Cohen, S., Park, T. G., Gupta, R. K., Siber, G. R., Langer, R. (1993) Determinants of release rate of tetanus vaccine from polyester microspheres. *Pharm. Res.* **51**: 945–953

Alonso, M. J., Gupta, R. K., Min, C., Siber, G. R., Langer, R. (1994) Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. *Vaccine* **12**: 299–306

Barrera, D. A., Zylstra, E., Lansbury, P. T., Lancer, R. (1995) Copolymerization and degradation of poly(lactic-co-lysine). *Macromolecules* **28**: 425–432

Brunner, A., Mader, K., Gopferich, A. (1999) pH and osmotic pressure inside biodegradable microspheres during erosion. *Pharm. Res.* **16**: 847–853

Chang, A. C., Gupta, R. K. (1996) Stabilization of tetanus toxoid in poly (DL-lactic-co-glycolic acid) microspheres for the controlled release of antigen. *J. Pharm. Sci.* **85**: 129–132

Cleland, J. L., Jones, A. J. S. (1996) Stable formulations of recombinant human growth hormone and interferon-gamma for microencapsulation in biodegradable microspheres. *Pharm. Res.* **13**: 1464–1475

Crotts, G., Park, G. (1998) Protein delivery from poly (lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues. *J. Microencapsul.* **15**: 699–713

Eldridge, J. H., Staas, J. K., Meulbroek, J. A., Tice, T. R., Gilley, R. M. (1991) Biodegradable and biocompatible poly (DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. *Infect. Immun.* **59**: 2978–2986

Esparza, I., Kissel, T. (1992) Parameters affecting the immunogenicity of microencapsulated tetanus toxoid. *Vaccine* **10**: 714–720

Heller, J. (1990) Development of poly (orthoesters): a historical overview. *Biomaterials* **11**: 659–665

Igartua, M., Hernandez, R. M., Esquisabel, A., Gascon, A. R., Calvo, M. B., Pedraz, J. L. (1998) Enhanced immune response after subcutaneous and oral immunization with biodegradable PLGA microspheres. *J. Control. Release* **56**: 63–73

Jameela, S. R., Amit, M., Jayakrishnan, A. (1994) Cross-linked chitosan microspheres as carriers for prolonged delivery of macromolecular drugs. *J. Biomater. Sci.* **6**: 621–632

Laemmli, K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**: 680–685

Mehta, R. C., Jeyanthi, R., Calis, S., Thanoo, B. C., Burton, K. W., De Luca, P. P. (1994) Biodegradable microspheres as depot system for parenteral delivery of peptide drugs. *J. Control. Release* **29**: 375–384

Morlock, M., Kroll, H., Winter, G., Kissel, T. (1997) Microencapsulation of rh-erythropoietin using biodegradable poly (D,L-lactide-co-glycolide): protein stability and the effect of stabilizing excipients. *Eur. J. Pharm. Biopharm.* **43**: 29–36

Nellore, R. V., Pande, P. G., Young, D., Bhagat, H. R. (1992) Evaluation of biodegradable microspheres as vaccine adjuvant for hepatitis B surface antigen. *J. Parenter. Sci. Technol.* **46**: 176–180

O'Hagan, D. T., Jeffery, H., Davis, S. S. (1993) Long term antibody responses in mice following subcutaneous immunization with ovalbumin entrapped in biodegradable microspheres. *Vaccine* **11**: 965–969

Okada, H., Toguchi, H. (1995) Biodegradable microspheres in drug delivery. *Crit. Rev. Ther. Drug Carrier Syst.* **12**: 1–99

Park, T. G., Lu, W., Crotts, G. (1995) Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly (D,L-lactic-co-glycolic acid) microspheres. *J. Control. Release* **33**: 211–222

Perez, C., Griebenow, K. (2001) Improved activity and stability of lysozyme at the water/CH_2Cl_2 interface: enzyme unfolding and aggregation and its prevention by polyols. *J. Pharm. Pharmacol.* **53**: 1217–1226

Perez, C., Castellanos, I. J., Costantino, H. R., Al-Azzam, W., Griebenow, K. (2002) Recent trends in stabilizing proteins structure upon encapsulation and release from bioerodible polymers. *J. Pharm. Pharmacol.* **54**: 301–313

Putney, S. D., Burke, P. A. (1997) Improving protein therapeutics with sustained-release formulations. *Nat. Biotechnol.* **16**: 153–157

Ryan, E. J., Daly, L. M., Mills, K. H. G. (2001) Immunomodulators and delivery systems for vaccination by mucosal routes. *Trends Biotechnol.* **19**: 293–304

Shi, L., Caulfield, M. J., Chern, R. T., Wilson, R. A., Sanyal, G., Volkin, D. B. (2002) Pharmaceutical and immunological evaluation of a single-shot hepatitis B vaccine formulated with PLGA microspheres. *J. Pharm. Sci.* **91**: 1019–1035

Singh, M., Li, X. M., Mc Gee, J. P., Zamb, T., Koff, W., Wang, C. Y., O' Hagan, D. T. (1997) Controlled release microparticles as a single dose hepatitis B vaccine: evaluation of immunogenicity in mice. *Vaccine* **15**: 475–481

Zhu, G., Mallery, S. R., Schwendeman, S. P. (2000) Stabilization of proteins encapsulated in injectable poly (lactide-co-glycolide). *Nat. Biotechnol.* **18**: 52–57