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Influence of surface charge of PLGA particles of recombinant hepatitis B surface antigen in enhancing systemic and mucosal immune responses

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ABSTRACT

This study investigates the efficacy of surface-modified microspheres of hepatitis B surface antigen (HBsAg) in eliciting systemic and mucosal immune responses. Positively charged poly(D,L-lactic-*co*-glycolic acid) microspheres were prepared by a double-emulsion solvent-evaporation method with cationic agents—stearylamine and polyethylenimine—in the external aqueous phase. Formulations were characterized for morphology, size, density, aerodynamic diameter, entrapment efficiency and in vitro drug-release profile. Immunization was performed after pulmonary administration of the formulations to female Sprague–Dawley rats and the immune response was monitored by measuring IgG levels in serum and secretory (sIgA) levels in salivary, vaginal and bronchoalveolar lavage fluids. The cell-mediated immune response was studied by measuring cytokine levels in spleen homogenates, and a cytotoxicity study was performed with Calu-3 cell line. The aerodynamic diameter of the particles was within the respirable range, with the exception of stearylamine-modified particles. Zeta potential values moved from negative (−6.76 mV) for unmodified formulations to positive (+0.515 mV) for polyethylenimine-modified particles. Compared to unmodified formulations, polyethylenimine-based formulations showed continuous release of antigen over a period of 28–42 days and increased levels of IgG in serum and sIgA in salivary, vaginal and bronchoalveolar lavage. Further, cytokine levels—interferon γ and interleukin-2—were increased in spleen homogenates. The viability of Calu-3 cells was not adversely affected by the microparticles. In summation, this study establishes that positive surface charges on poly(D,L-lactic-*co*-glycolic acid) particles containing HBsAg enhances both the systemic and mucosal immune response upon immunization via the respiratory route.

1. Introduction

Over the past decade, mucosal vaccination has received considerable interest as a non-invasive and safe alternative to injectable vaccines. One of the reasons for increased attention to mucosal routes is that the majority of diseases are transmitted through mucosal membranes. Indeed, mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts are the main ports of entry for many pathogens. Antibodies produced by mucosal membranes include secretory IgA (sIgA), which are very stable, have high affinity for mucosal surfaces, and act as the organism's first line of defense to neutralize invading pathogens and prevent their adhesion to the epithelial cell lining (Rosenthal and Gallichan, 1997; McCluskie and Davis, 1999; Ogra et al., 2001; Jaganathan and Vyas, 2006). Furthermore, lymphocytes stimulated by antigens at the mucosal site can migrate via lymph nodes and the thoracic duct to distal mucosal effector sites and via the circulatory system, thereby inducing both systemic and mucosal immune responses (Nagler-Anderson, 2001; Azzali, 2003; MacDonald, 2003; Bivas-Benita et al., 2005; Lu and Hickey, 2007). Moreover, most of the currently available vaccines uses alum as adjuvant, which is poorly immunogenic (Ma et al., 2007) and produces only humoral immunity but fails to produce cell-mediated immunity, the latter being an important mechanism for effective clearance of viral infections. Moreover, being needle-free, mucosal vaccination is likely to reduce the transmission of infectious agents such as hepatitis B virus and HIV.

In truth, a mucosal vaccine against hepatitis B infection would be an ideal delivery approach because the disease is transmitted by direct contact with mucous membranes (Mestecky, 1987; Cui and Mumper, 2002). Recently, we showed that recombinant hepatitis B vaccine formulated with tetradecyl maltoside, a non-ionic surfactant, elicits an immune response upon administration via the pulmonary route (Thomas et al., 2008). This adjuvant is believed to produce increases in antibody levels by facilitating interactions between the antigen and antigen presenting cells located in the respiratory epithelium. However, this type of antigen formulation failed to maintain a sustained immune response. In a separate study,

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we showed that it is feasible to encapsulate a non-peptide drug (low molecular weight heparin) or recombinant hepatitis B surface antigen (HBsAg) in poly(D,L-lactic-*co*-glycolic acid) (PLGA) particles such that the resulting particles produce continuous release of the drug or antigen upon inhalation by rats (Rawat et al., 2008; Thomas, 2009). However, dose-to-dose variability and lack of reproducibility in delivering a correct dose remain as major limitations of vaccine delivery via the respiratory route. In fact, erratic deposition of aerosolized particles carrying antigens and subsequent poor internalization of the antigens are believed to be the reasons for variability associated with vaccination via oral inhalation. The deposition pattern of particles in the lungs is dictated by the aerodynamic diameter of particles or inhalable formulations. Particles with an aerodynamic diameter of 1–5 μm are considered optimal for deposition in the alveolar region (Patton, 1996; Edwards et al., 1997; Patton et al., 2004; Amidi et al., 2007; Amorij et al., 2007).

The problems of suboptimal deposition in the respiratory tract and poor antigen presentation can be overcome by encapsulating the antigen in positively charged particles with an aerodynamic diameter between 1 and 5 μm (Amidi et al., 2007). Indeed, cationic particles or microparticles with positive surface charge have been shown to produce a stronger immune response compared to negatively charged or neutral particles (Jaganathan and Vyas, 2006). Cationic microparticles are known to be more efficiently internalized by macrophages and dendritic cells (Singh et al., 2000; Thiele et al., 2003; Wischke et al., 2006). For example, DNA vaccine formulated in positively charged particles is reported to induce strong cytotoxic T-lymphocyte responses after intramuscular administration (Singh et al., 2000; Wischke et al., 2006). PLGA microspheres containing a variety of antigens have been shown to increase the immune response upon administration via various mucosal routes (Nellore et al., 1992; Shi et al., 2002; Jaganathan et al., 2004; Feng et al., 2006; Jaganathan and Vyas, 2006). However, currently there are no studies regarding the use of PLGA particles with positive surface charge in delivering recombinant HBsAg via the pulmonary route. Importantly, it is still unknown whether inhalation of porous PLGA microspheres of HBsAg produces mucosal and cell-mediated immune responses in addition to a humoral immune response as observed in our earlier study (Thomas, 2009). The influence of surface charge in eliciting mucosal and systemic immune responses upon pulmonary administration of HBsAg also remains to be elucidated. Therefore, in the present study, we prepared positively charged, inhalable porous PLGA microspheres of HBsAg and investigated the efficacy of these surface-modified carriers in generating systemic, mucosal and cell-mediated immune responses upon aerosolization of the particles to the lungs.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-*co*-glycolide) (PLGA) polymer having a 85:15 ratio with inherent viscosity 0.55–0.75 dL/g (average molecular weight = 85.2 kDa) and a 50:50 ratio with inherent viscosity 0.55–0.75 dL/g (average molecular weight = 43.5 kDa) was purchased from Boehringer Ingelheim (Lactel Absorbable Polymers, Pelham, AL). HBsAg in phosphate buffer at a concentration of 2 mg/ml was a gift of Shantha Biotechnics (Hyderabad, India). Polyvinyl alcohol (PVA), horseradish-conjugated goat anti-rat IgA, and IgG were purchased from Sigma (Sigma–Aldrich Inc., St. Louis, MO). Bicinchoninic acid assay (BCA) and microBCA protein assay kits were procured from Pierce Biotechnology (Rockford, IL).

Table 1
Composition and formulation codes of HBsAg-entrapped PLGA microspheres.

Formulations	IAP:OP:EAP (v/v/v)	OP (PLGA)	EAP
SMP-1	0.250:5:25	85:15	5% PVA
SMP-2	0.250:5:25	50:50	5% PVA
SMP-3	0.250:5:25	85:15	5% PVA + 0.5% SA
SMP-4	0.250:5:25	85:15 PLGA + 0.5% SA	5% PVA
SMP-5	0.250:5:25	85:15	5% PVA + 0.5% PEI
SMP-6	0.250:5:25	50:50	5% PVA + 0.5% PEI

2.2. Preparation of surface-modified, HBsAg-entrapped PLGA microparticles

HBsAg-entrapped PLGA microspheres (Table 1) were prepared by the water-in-oil-in-water (w/o/w) emulsion and evaporation method using PLGAs of two different molecular weights (Edwards et al., 1997). To prepare conventional microspheres, 250 μl of recombinant HBsAg solution (internal aqueous phase; IAP) containing 2% (w/v) sucrose and 2% (w/v) $Mg(OH)_2$ as protein stabilizers was first emulsified in 5.0 ml of dichloromethane (organic phase: OP) containing the polymer (0.250 g) with a probe sonifier (Branson Sonifier 450; Branson Ultrasonics Corporation, Danbury, CT) for 60 s in an ice-bath (20 W, 6 cycles of 10 s each at 40% duty cycle). The resulting water-in-oil (w/o) primary emulsion was then poured into 25 ml of 5.0% (w/v) aqueous solution of PVA (external aqueous phase, EAP) and emulsified by homogenization for 15 min at 15,000 rpm. Surface-modified microspheres were prepared by incorporation of 0.5% stearylamine (SA) or polyethylenimine (PEI) in the EAP. The secondary emulsion was then stirred overnight at room temperature for evaporation of the organic phase. The resulting polymeric particles were washed thrice and lyophilized to get free-flowing microparticles. Blank microspheres without HBsAg were also prepared following the same procedure. Each batch was prepared in triplicate.

2.3. Characterization of particles

Scanning electron microscope (SEM) images of different formulations were obtained to study the morphology and the surface characteristics of the PLGA microspheres (Hitachi S-3400N, Freehold, NJ). For SEM studies, PLGA microspheres were mounted on aluminum stubs using self-adhesive carbon disks and then sputter-coated with a conducting layer of gold under argon (Emitech K550X, Kent, UK). The images were taken at a voltage of 9–16 kV. *The mean volume geometric diameter* and particle size-distribution of the microspheres were determined by a laser light-scattering technique using a Microtrac® S3500 (North Largo, FL). For the particle size analysis, the microspheres were dispersed in a 0.2% (w/v) solution of Tween 80. Particle size analysis was performed in triplicate and expressed as volume mean diameter ± SD. *Powder density* was estimated from tapped density as described previously (Rawat et al., 2008). An aliquot (100 mg) of microspheres was transferred to a 10 (±0.05) ml graduated cylinder and the initial volume was recorded. The cylinder containing the microspheres was then tapped 200 times and the volume of the particles was again recorded. The tapped density of particles (ρ) was calculated from the ratios of the volumes (ml) occupied before and after 200 taps. The theoretical mass mean aerodynamic diameter (MMADt) of the particles was calculated using the following equation, as reported earlier (Ungaro et al., 2006; Rawat et al., 2008):

$$\mathrm{MMADt} = d\left(\frac{\rho}{\rho_0 X}\right)^{1/2}$$

where d is the geometric mean diameter, ρ is the particle mass density, ρ_0 is a reference density of 1 g/cc, and X is the dynamic shape factor, which is 1 for a sphere.

The *zeta potential* of surface-modified PLGA microspheres were measured using a NICOMP™ 380 ZLS instrument (PSS NICOMP, Santa Barbara, CA). For zeta potential measurements the HBsAg-entrapped PLGA formulations were dispersed in de-ionized water containing 1 mM potassium chloride solution as previously reported (Gutierro et al., 2002). Each sample was tested in triplicate and the data are reported as mean ± SD.

The amount of antigen loaded in PLGA microspheres was estimated by using the BCA method (Pierce, Rockford, IL). Briefly, 10 mg of the HBsAg-encapsulated PLGA microspheres were dissolved in 1 ml of acetonitrile, vortexed and centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was removed carefully and the precipitate was redissolved in 1 ml solution of 1% sodium dodecyl sulfate (SDS). The resulting solution was then used to estimate the HBsAg content by the BCA method. Blank microspheres without HBsAg and containing only sucrose and $Mg(OH)_2$ were used as a control. The actual drug loading was calculated by taking into account the amount of the polymer used in the preparation of the microspheres. The entrapment efficiency of the formulations was expressed as the percentage of calculated antigen loaded in the microspheres compared with the actual amount of the antigen added during the preparation (Rawat et al., 2008).

2.4. In vitro release experiments

The in vitro release studies were performed in phosphate buffer. An aliquot (20 mg) of freeze-dried microspheres of HBsAg was suspended in micro-centrifuge tubes containing 1 ml of phosphate-buffered saline (PBS; pH 7.4). The samples were incubated at 37 °C under gentle shaking (150–200 rpm), and at pre-determined time points the vials were removed from the incubator and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was collected, and mixed with PBS, and finally the antigen was quantitated by BCA assay. The in vitro release studies were conducted over 42 days and the release profiles are reported as cumulative antigen released against time.

2.5. Pulmonary immunization studies

Immunization studies were performed according to our previously published method (Thomas et al., 2008). Prior to the experiment, female Sprague–Dawley rats (Charles River Laboratories, Charlotte, NC) weighing between 150 and 200 g were anesthetized by an intramuscular (IM) injection of an anesthetic cocktail containing xylazine (20 mg/ml) and ketamine (100 mg/ml) and divided into six groups (8–10 rats in each group) to receive the following six treatments: (i) no treatment; (ii) blank microspheres with no antigen; (iii) IM plain HbsAg; (iv) inhaled plain HBsAg; (v) unmodified PLGA containing HBsAg, SMP-1; and (vi) PEI-modified microspheres containing HBsAg. The formulations used in the in vivo studies were selected based on the results of the in vitro characterization studies described above. The dose of HBsAg administered was 10 μg/kg and all treatment groups received the dose of antigen on days 0 and 14. The formulations were administered as aerosols to the lungs by a Microsprayer™ attached to a syringe (Penn-Century, Inc., PA) as reported by us previously (Thomas et al., 2008).

2.6. Evaluation of immune response

The humoral immune response was evaluated by determining serum IgG levels. Blood samples were collected on days 7, 14 and 28 after pulmonary or intramuscular administration of the formulations. Briefly, the animals were anesthetized by using an anesthetic cocktail as described above and blood was collected from the tail. The collected samples were centrifuged at 6000 rpm for 10 min in a microentrifuge tube (Eppendorf AG, Hamburg, Germany) and the supernatant serum was collected and stored at −20° C until further analysis. Specific antibodies generated to HBsAg were analyzed by using a commercially available ELISA kit (HBsAb, Immuno Diagnostics, Foster City, CA) as per our previously published procedure (Thomas et al., 2008).

To study the mucosal immune response, secretory antibody levels (sIgA) in vaginal, salivary and bronchoalveolar lavage (BAL) fluids were quantified. The vaginal and salivary secretions were collected at three time periods: (i) day 0, prior to immunization; (ii) day 28, four weeks after the first immunization; and (iii) day 42, at the end of the six-week study period. The vaginal washes and salivary secretions were collected according to a previously published method (Debin et al., 2002; Jaganathan and Vyas, 2006). For collection of salivary secretion, an approximately 10 mg/ml sterile solution of pilocarpine was administered intraperitonially (0.1–0.2 ml, depending on the weight of the rat). Within a few minutes of injection, the rats begin to salivate and the saliva was collected using a pipette. For collection of vaginal wash, about 50 μl of 1% (w/v) bovine serum albumin (1% BSA–PBS) was introduced into the vaginal tract of anesthetized rats using a pipette. The solution was withdrawn and reintroduced nine times and the final wash was stored at −20 °C. The following day, a second vaginal wash was collected by following the same procedure and pooled with the first sample. For collection of BAL fluid, animals were sacrificed on day 42 and the BAL fluid was obtained according to our published procedure (Thomas et al., 2008). In brief, the respiratory apparatus was exposed by a mid-level incision in the thoracic cavity of the anesthetized animal. After exsanguination by severing the abdominal aorta, the lungs were surgically removed and lavaged by instilling a 5-ml aliquot of normal saline into the trachea. The instilled saline was left in the lungs for 30 s, withdrawn, re-instilled for an additional 30 s and then finally withdrawn into a centrifuge tube. The lavage fluid obtained was centrifuged at 500 × g for 10 min and the supernatant was collected. All the samples were stored at −20 °C for further analysis. Secretory antibody levels (sIgA) were determined using a commercially available ELISA kit (Bethyl Laboratories, Montgomery, TX).

2.7. Evaluation of endogenous cytokine levels

The cell-mediated immune response was evaluated by measuring cytokine levels—interferon-γ (IFN-γ) and interleukin-2 (IL-2)—in spleen homogenates according to a published procedure (Vyas et al., 2005). Spleens were removed after euthanizing the animals at the end of immunization study. The spleens were weighed and 10% w/v homogenates were prepared by homogenizing the spleen in ice-cold 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma, St. Louis, MO) prepared in PBS. The homogenates thus obtained was incubated for 2 h in an ice-bath to allow the insoluble matter to settle. The supernatant was centrifuged at ∼2000 × g for 20 min and the endogenous cytokines (IL-2 and IFN-γ) in the supernatant were assayed using Duoset sandwich ELISA kits (R&D systems, Minneapolis, MN) in accordance with the manufacturer's instructions.

All animal studies were approved by the Texas Tech University Health Sciences Center (TTUHSC) Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Protocol # 02004).

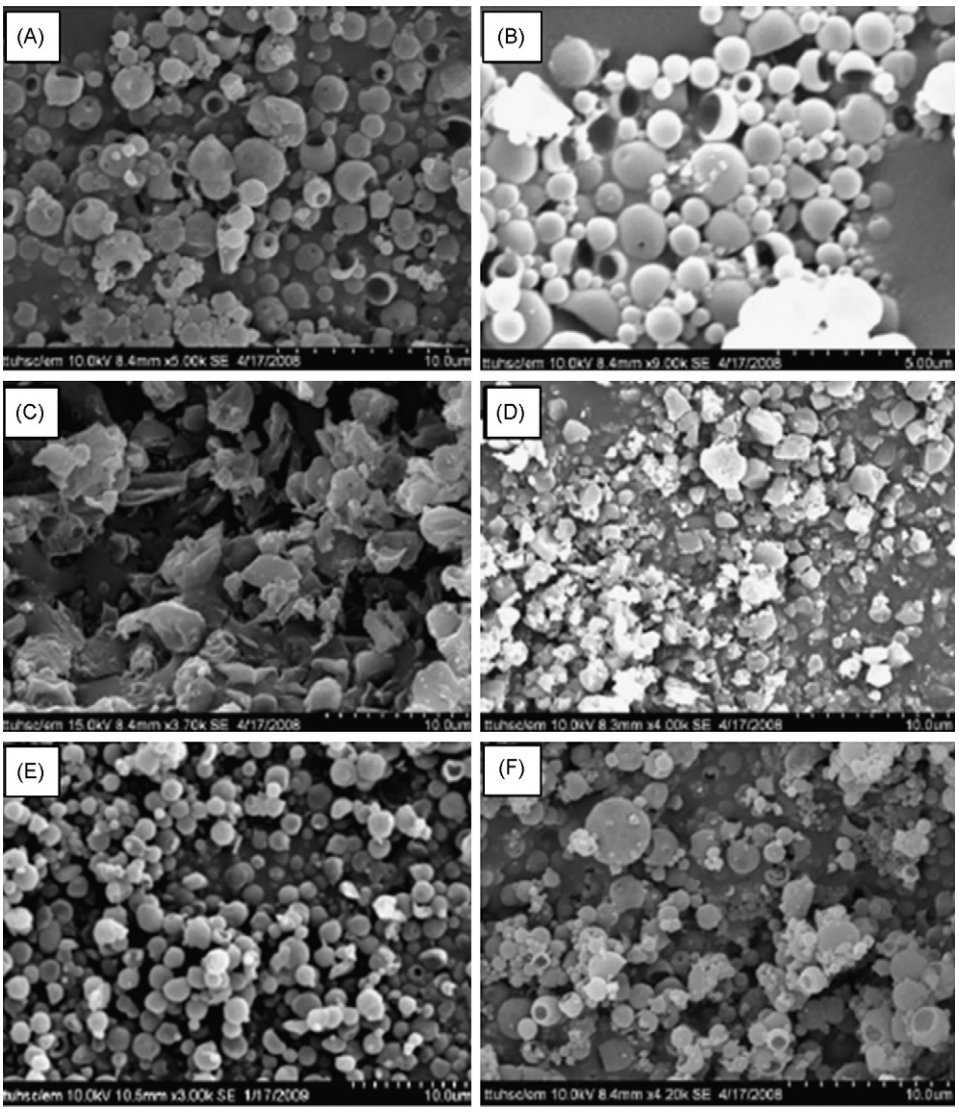

Fig. 1. Surface morphology of HBsAg-loaded surface-modified PLGA microspheres; Images of particles of (A) SMP-1, (B) SMP-2, (C) SMP-3, (D) SMP-4, (E) SMP-5, and (F) SMP-6 viewed under the scanning electron microscope. Refer to Table 1 for compositions of the different formulations.

2.8. Cytotoxicity studies of PLGA microspheres in the Calu-3 cell line

The safety of PLGA particles was studied by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) using the human bronchial epithelial cell line, Calu-3. The toxicity of two formulations, SMP-1 and SMP-5, was studied; blank PLGA microspheres without surface-modifying agents and PEI alone were used as controls. The concentrations of PLGA and PEI were 0.5, 2.5, 5, and 10 mg/ml, respectively. Saline and sodium dodecyl sulfate (SDS, 0.1%) were used as negative and positive controls, respectively. The cells were plated into 96-well micro-titer tissue culture plates at a density of 50,000 cells per well and incubated at 37 °C in 5% CO_2 and 90% relative humidity. Cell viability was measured by the MTT assay as previously described (Rawat et al., 2008). Briefly, 4 h after incubation with 20-μl test formulations, 20 μl of MTT (5 mg/ml) solution was added to each well and the cells were incubated at 37 °C for another 4 h. The solution in each well was then carefully aspirated and 100 μl of DMSO was added. Finally, the plates were incubated again at 37 °C for 1 h. Each formulation was tested in 12–16 wells (n = 12–16). Optical density of the wells was measured on a micro-titer-plate reader (TECAN U.S. Inc., Research Triangle Park, NC) at 570 nm. Cell viability was expressed as the percentage absorbance of test compound and controls relative to DMEM medium alone.

2.9. Statistical analysis

One-way ANOVA was used to compare the data. When the differences in the means were significant, post hoc pair wise comparisons were conducted using Tukey-Kramer multiple comparison tests with GraphPad InStat™ software (GraphPad Software, San Diego, CA). p-values of less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Physical characterization of surface-modified PLGA microspheres

The morphology of plain and surface-modified PLGA microspheres was examined by scanning electron microscopy. Images of plain microspheres without any surface-modifying agents showed particles with smooth surfaces and occasionally particles with empty cores (Fig. 1A and B). The smooth surfaces and uniform size-

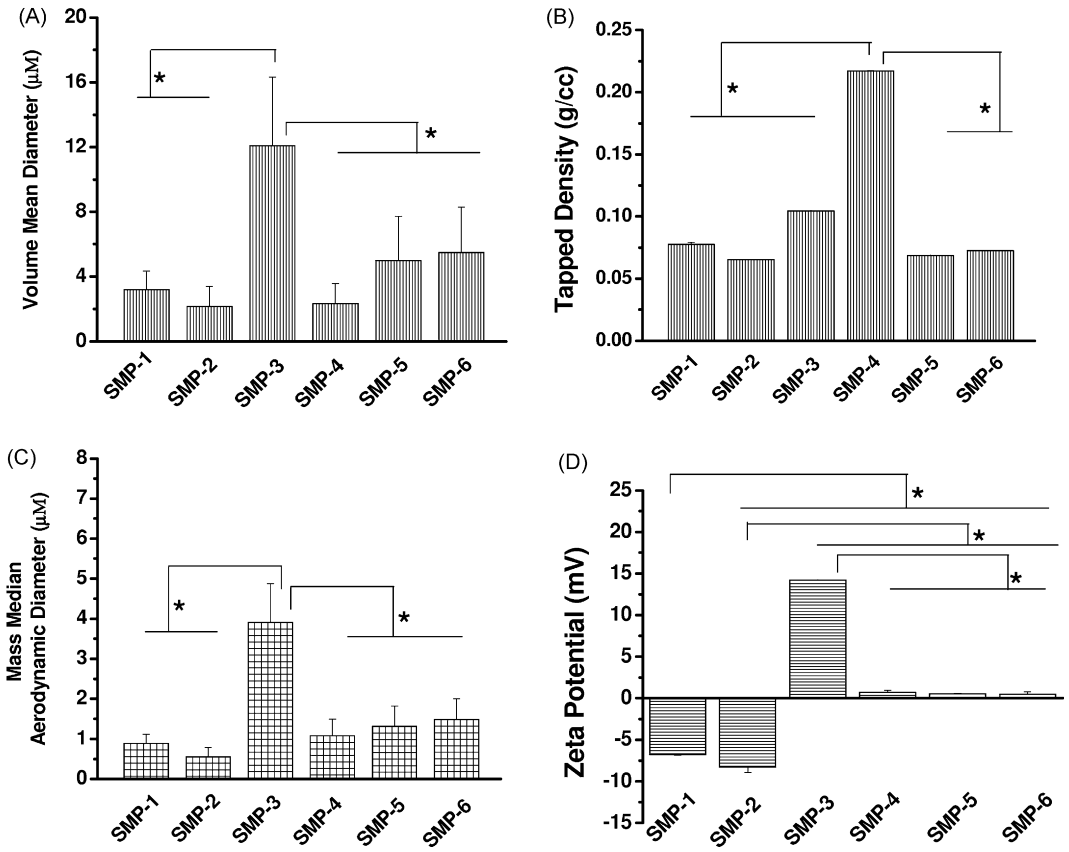

Fig. 2. Physical characterization of HBsAg-loaded PLGA microspheres. (A) Volume-based mean diameter; (B) tapped density; (C) theoretical mass median aerodynamic diameter (MMADt); (D) zeta potential. Data represent mean ± SD ($n = 3$). *Results are significantly different, $p < 0.05$.

distribution of the particles may be attributed to the use of 5% PVA, which acts as an emulsion stabilizer, preventing coalescence of the emulsion droplets (Lee et al., 1999; Yang et al., 2001). However, incorporation of stearylamine (SA) in SMP-3 and SMP-4 disrupted the formation of the emulsion, resulting in agglomerations of particles rather than free-flowing individual particles (Fig. 1C). This is because SA is very hydrophobic and practically insoluble in water (Rawat et al., 2008). When stearylamine was incorporated into the organic phase, the surface of SA-modified particles became a little smoother and the particle sizes were more evenly distributed compared to particles without SA. However, these particles still did not attain complete uniformity of shape and size (Fig. 1D) when compared to the other formulations. Unlike SA-modified particles, PEI-modified microparticles had smooth surfaces and were of uniform shape and size (Fig. 1E and F). Previously we observed similar uniformly sized PEI-modified PLGA particles; this uniformity occurs because PEI is very hydrophilic and facilitates the formation of homogeneous droplets of emulsion.

The surface-modified microspheres were also characterized for particle size, tapped density, theoretical aerodynamic diameter (MMADt), and zeta potential. All particles were 1–5 μm in size, except the SA-modified particles, which formed agglomerates (Fig. 2A). Due to excessive agglomeration, the geometric diameter of the SMP-3 microspheres was ~12 μm. The type of PLGA polymer (85:15 or 50:50) used did not have any significant effect on the size of the microspheres.

The tapped density of all formulations ranged between 0.065 and 0.078 g/cc, suggesting that all but the SA-modified particles were highly porous (Fig. 2B). The SA-modified formulations, however, showed higher tapped densities, 0.1–0.2 g/cc (SMP-3 and SMP-4). This may be due to irregularities in the size and shape of the particles in these formulations, as evident from the SEM analysis. As seen in Fig. 2C, theoretical mass median aerodynamic diameter (MMADt) values of all the particles were near the critical 1–5 μm size range. That the SMP-3 formulation showed MMADt in the respirable range despite having a larger particle size is because the density of these particles was lower than that of the SMP-4 formulation. However, because the theoretical MMAD takes only the size and tapped density into account, the actual MMAD might be quiet different because particle shape also plays an important role in determining the experimental MMAD. In the case of vaccine delivery, geometric diameter or particle size is the main parameter that influences the robustness of the immune response. In fact, particles in the aerodynamic size range of 1–5 μm are considered ideal for

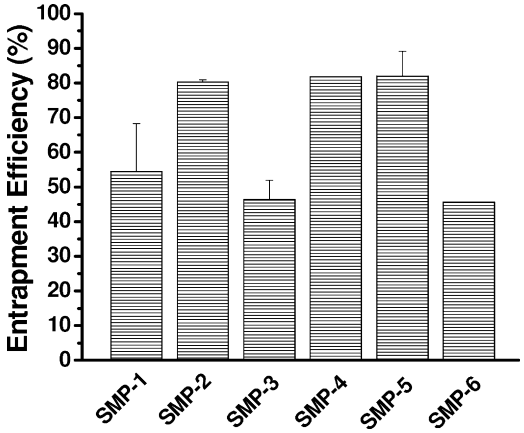

Fig. 3. Entrapment efficiency of HBsAg-loaded PLGA microspheres. Data represent mean ± SD ($n = 3$).

pulmonary vaccination (Amidi et al., 2007; Amorij et al., 2007). We also showed previously that particles <10 μm are more efficiently taken up by macrophages compared to particles larger than 10 μm size (Thomas, 2009). The data presented in Fig. 2D show that plain PLGA microspheres (SMP-1 and SMP-2) were negatively charged. However, upon surface modification with SA and PEI, the particles became positively charged. Only the SMP-3 particles showed a significantly high positive surface charge (~14 mV), whereas the other surface-modified formulations showed zeta potential values in the range of +0.51 to +0.735 mV. The high positive surface charge of the SMP-3 formulation may stem from the fact that a significant portion of SA used in these formulations remained on the surface of the particles because of the chemical's poor solubility in the aqueous phase. The change in sign of the zeta potential is due to incorporation of the positively charged agents SA and PEI during the double emulsification process.

3.2. Entrapment efficiency

The entrapment efficiency of HBsAg antigen loaded into the PLGA microspheres was determined by using a microBCA protein assay kit. The entrapment efficiency was 50 to 80% for plain particles (SMP-1 and SMP-2) with no surface modification (Fig. 3). The entrapment efficiency of SMP-2 was higher than that of SMP-1 perhaps because there were more empty cores in the former formulation, as observed under SEM. The entrapment efficiency was significantly decreased when SA was added to the EAP as a surface-modifying agent, which may be a result of the poor solubility of SA in the aqueous phase. As SA does not form a homogeneous solution upon incorporation in the EAP, it makes the emulsion unstable and eventually leads to a flaky or coalesced formulation. However, when SA was added to the organic phase (SMP-4), an increase in entrapment efficiency was observed compared to particles prepared by adding SA to the EAP (SMP-4). This increased entrapment efficiency could be because SA is soluble in dichloromethane and thus helps to form a stable primary emulsion. Another explanation is that SA, a cationic surfactant, forms a complex with the negatively charged PLGA and HBsAg, helping to stabilize the formulation and subsequently increasing the entrapment efficiency (Rawat et al., 2008). Similarly, a significant increase in entrapment efficiency was observed in PEI-modified particles. Compared to its unmodified counterpart (SMP-1), PEI-modified particles (SMP-5) showed an ~30% increase in entrapment efficiency (Fig. 3). The higher entrapment efficiency of PEI-modified formulations was because of the increased stability of the w/o/w emulsion due to the presence of the polycation PEI. The increase in entrapment efficiency can also be explained by an electrostatic interaction between PEI and PLGA that enables HBsAg become entrapped in the PLGA-PEI polymeric matrix to a larger extent. Similar data have been obtained for incorporation of DNA and proteins within alginate/chitosan matrices (Wee and Gombotz, 1998; Li et al., 2008). However, when a more hydrophilic PLGA was used, a reduction in entrapment efficiency was observed even in the presence of PEI in the EAP (SMP-6).

3.3. In vitro release profiles

The release profiles of all formulations were studied in PBS. The amount of antigen released was determined by using a microBCA protein assay kit as mentioned in Section 2. The amount of antigen released (% cumulative release) at time zero was considered as surface-associated antigen, whereas the amount of antigen released at the end of 1 h was considered as the initial burst phase of antigen release (Rawat et al., 2008). The SMP-1, SMP-3 and SMP-6 formulations showed higher cumulative releases, with nearly 80-100% of antigen released at the end of 28 days (Fig. 4A). The SMP-2, SMP-4 and SMP-5 formulations showed a lower cumulative release,

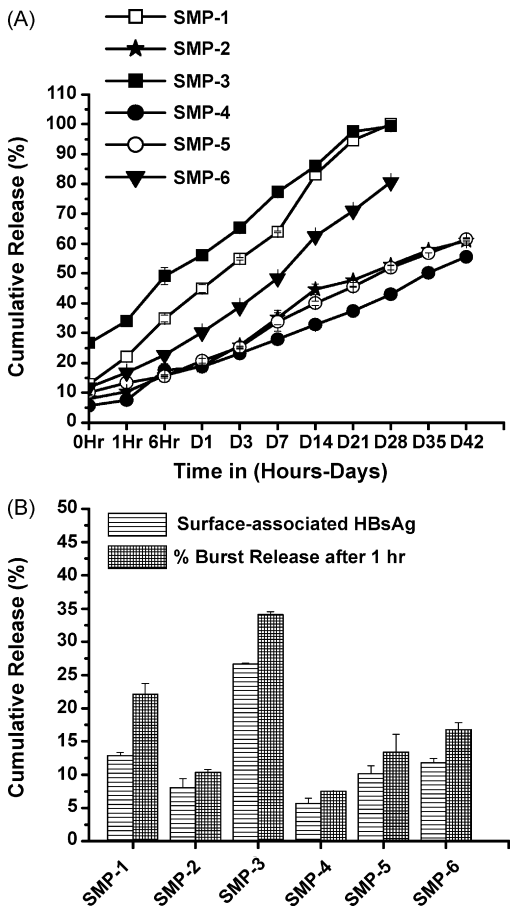

Fig. 4. In vitro release profiles of HBsAg-loaded PLGA microspheres (A) cumulative release profiles for 42 days and (B) burst and surface-associated release. Data represent mean ± SD ($n = 3$).

however, the release was continuous and lasted up to 42 days. These differences in release pattern of the formulations may be due to a combination of factors, such as entrapment efficiency, type of PLGA polymer, particle size, and biodegradability of the formulations. Although the PLGA used to prepare the SMP-1 formulation was more hydrophobic than that used in the SMP-2 formulation, antigen release from the latter was slower. The reason for this anomalous release pattern is not known, although we believe that either the smaller particle size of SMP-2 or some process parameter might have been a factor. It is also possible that this formulation may have undergone some form of degradation during preparation; hence, the SMP-2 formulation was removed from further studies.

SMP-3 showed both the highest surface-associated antigen and the highest initial burst release (Fig. 4B), perhaps because the addition of SA produced agglomerates of particles instead of free-flowing particles, as observed under SEM (Fig. 1C). Indeed, all surface-modified particles, except SMP-3, showed a slower release compared to unmodified particles. The amount of surface-associated drug and the initial burst release from these formulations were not significantly different. SMP-5 and SMP-6 showed a slightly higher burst release (13.38 ± 2.74) compared to SMP-4. This slight difference between the release profiles of SA-modified particles (SMP-4) and PEI-modified particles can be explained by the fact that PEI is more hydrophilic than SA (Fig. 4B). Furthermore, particle size may also play a role in the release of the drug, because size is reported to be directly proportional to the amount of antigen released (Yang et al., 2001). This assumption agrees with the fact that the particle sizes of SMP-5 and SMP-6 were slightly larger than that of SMP-4 and so the release of for-

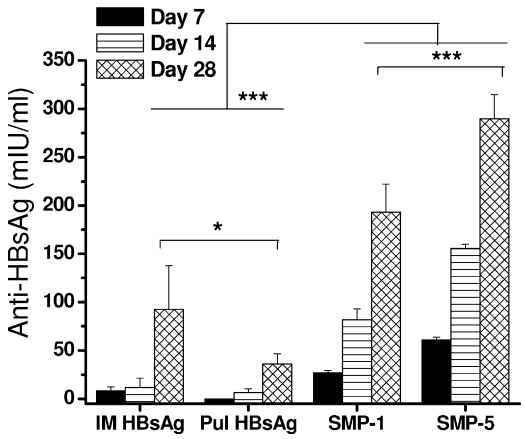

Fig. 5. Serum IgG levels on days 7, 14 and 28 after dosing of SMP-1 and SMP-5 formulations on days 0 and 14. Data represent mean ± SD, $n = 6–8$. *Results are significantly different, $p < 0.05$; **results are significantly different, $p < 0.01$; ***results are significantly different, $p < 0.001$.

mer two formulations were slightly higher than that produced by the latter one. Overall, based on the burst release, the formulations can be ranked (highest to lowest) as: SMP-3 > SMP-1 > SMP-6 > SMP-5 > SMP-2 > SMP-4 (Fig. 4B). Because the SMP-5 formulation showed a moderate burst phase release and a near-constant release of antigen over 42 days (Fig. 4A and B), this formulation was selected for the immune-response studies.

3.4. Evaluation of the immune response following pulmonary immunization

3.4.1. Serum antibody levels

The systemic immune response was studied by measuring HBsAg specific serum antibody levels. When plain HBsAg was administered by IM injection, a gradual increase in anti-HBsAg antibody levels was observed for 28 days (Fig. 5). Plain HBsAg administered via the pulmonary route (Fig. 5) showed an antibody profile similar to that elicited by IM vaccination, but with a reduced magnitude of immune response. Unlike plain IM or pulmonary HBsAg, unmodified microparticle formulation (SMP-1) administered by inhalation showed a continual increase in immune response for 28 days (Fig. 5). This increase in the serum antibody levels was found to be statistically significant compared to that produced upon administration of plain HBsAg via IM or pulmonary route ($p < 0.001$). However, when the microparticles with positive surface charge charge (SMP-5) was administered by the pulmonary route, it showed a significant increase in serum antibody levels compared to the plain IM, pulmonary HBsAg ($p < 0.001$) and the unmodified PLGA formulations (SMP-1; $p < 0.001$). Differences in the immune responses between SMP-1 and SMP-5 formulations can be explained by the differences in their surface charge. In fact, many published reports suggest that surface charge plays an important role on the immunogenicity of antigens entrapped in particulate carriers (Jaganathan and Vyas, 2006; Pandit et al., 2007). Based on these published reports, it is reasonable to argue that SMP-5, with a surface positive charge of +0.515 mV, may have been internalized by macrophages more efficiently than the unmodified negatively charged SMP-1 formulation having a zeta potential value of −6.76 mV. In addition to the surface positive charge, both the particles have particle diameter less than 10 μm which is further ideal for internalization by APCs, and hence both formulations have shown a high immune response after pulmonary administration. Other factors that may influence the generation of high antibody levels by particulate antigen include continuous processing and presentation of the antigen to the lymphocyte. These factors can also influence the magnitude of the immune response (Brewer et al., 2004; Fifis et al., 2004; Katare et al., 2005).

3.4.2. Mucosal immune responses

In this set of experiments, we tested the hypothesis that pulmonary immunization with HBsAg-entrapped PLGA microspheres induces a mucosal immune response in rats. Based on the in vitro release study, we selected two formulations for the mucosal immune-response study. The immune response produced by the formulations was compared with four controls: (i) no treatment, (ii) blank particles with no HBsAg, (iii) plain HBsAg administered via IM injection, and (iv) plain HBsAg administered via the pulmonary route. There was little or no increase in sIgA levels in the saliva of non-treated control rats. Plain HbsAg administered by IM or the pulmonary route also produced a negligible increase in sIgA levels in salivary secretions. The absence of a mucosal immune response is consistent with previous studies that showed negligible or no IgA response after IM immunization of HBsAg (Jaganathan and Vyas, 2006; Gupta et al., 2007). Administration of PLGA particles of HBsAg via the pulmonary route induced a mucosal immune response that was modest ($p > 0.05$). The greatest increase in sIgA levels was observed in rats that received PEI-modified microspheres via the pulmonary route (Fig. 6A). The differences between the levels of sIgA produced by the SMP-1 and SMP-5 formulations are attributed to the fact that SMP-5 was positively charged, which helped to facilitate internalization of the SMP-5 particles. The presence of a positive surface charge increases muco-adhesion by interacting with the negatively charged cell membrane, as proposed by others (Amidi et al., 2007). A similar increase in sIgA levels was observed when the mucosal immune response was measured in vaginal washes obtained on days 28 and 42 (Fig. 6B) and in BAL samples on day 42 (Fig. 6C). In all cases, the differences in sIgA levels elicited by PEI-modified particles compared to unmodified microspheres and control formulations were statistically significant $p < 0.05$. It is worth pointing out that sIgA is considered the main effector antibody for producing mucosal immunity. Induction of strong IgA antibody responses in distant mucosal sites such as the salivary and vaginal mucosae suggests that there is (i) effective targeting of lung lymphoid tissue, and (ii) dissemination of the mucosal response via cellular migration of primed antigen-specific B cells from the primary induction site, the lung (McDermott and Bienenstock, 1979; Smith et al., 2003; Amorij et al., 2007; Lu and Hickey, 2007).

3.5. Estimation of cytokine levels

Endogenous levels of cytokines IL-2 and IFN-γ were determined in spleen homogenates at day 42. Similar to the mucosal immune response profiles, there was no appreciable increase in cytokine levels in spleen homogenates of rats that received the four control treatments. Compared to rats that received plain IM HBsAg, significant increases in IL-2 and IFN-γ levels were observed in rats that received unmodified microspheres (SMP-1). The cytokine levels were further increased in rats that received PEI-modified microspheres (SMP-5) ($p < 0.05$). In fact, the SMP-5 formulation produced a 1.5- to 3-fold increase in cytokine levels compared to IM HBsAg and unmodified microspheres (Fig. 7). These data are consistent with published reports that suggest that a strong Th1-mediated cellular immune response is important for complete clearance of hepatitis B virus (Chisari and Ferrari, 1995; Constant and Bottomly, 1997; Vyas et al., 2005; Jaganathan and Vyas, 2006). This enhanced immune response can also be attributed to the fact that cationic microparticles are taken up more efficiently by dendritic cells and macrophages, as is observed with diethyl amino ethyl dextran (DEAEdextran)-based cationic microparticles (Wischke et al., 2006). Overall, the data presented in Figs. 5–7 suggest that PLGA

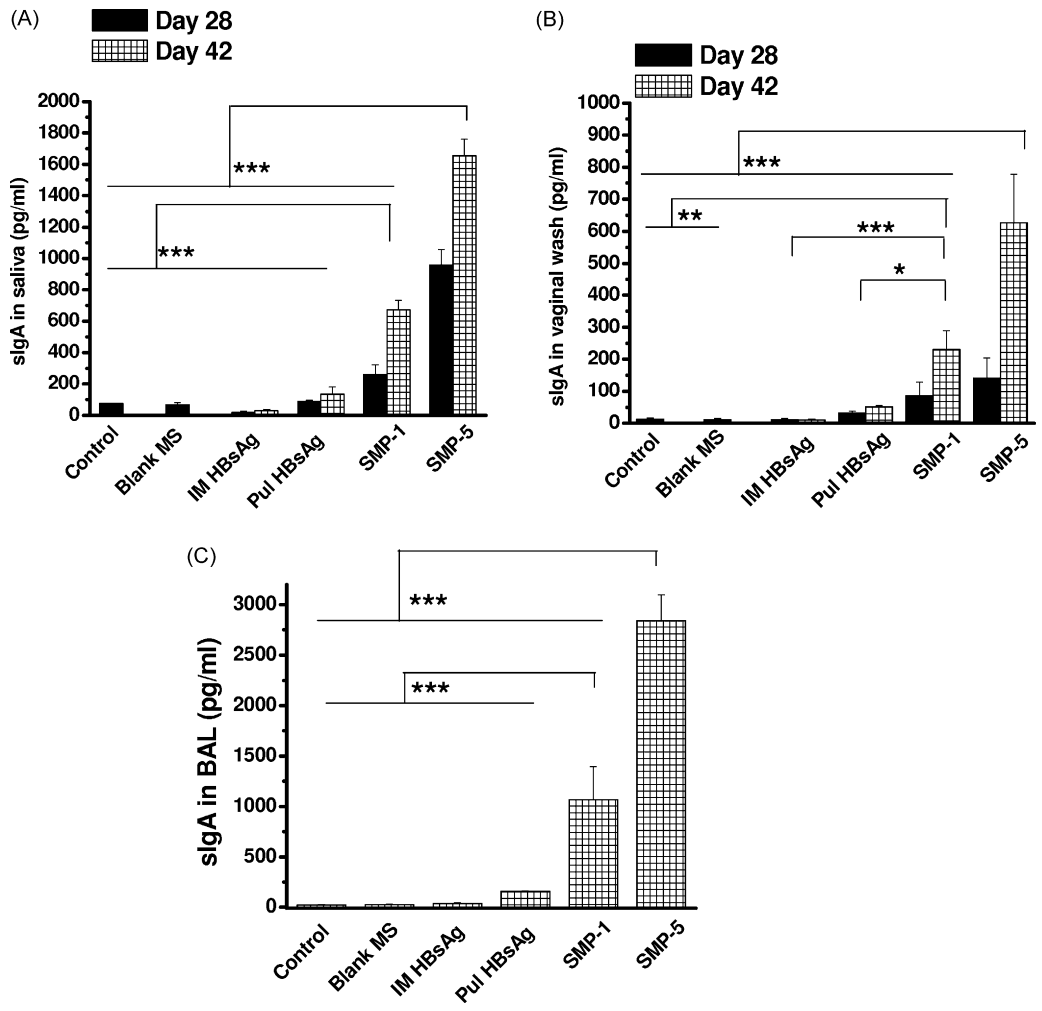

Fig. 6. Mucosal immune response profiles after 2 doses of vaccine or control formulation on days 0 and 14. Secretory IgA (sIgA) levels in (A) salivary secretion, (B) vaginal wash, and (C) broncholaveolar lavage (BAL) fluid. Data represent mean ± SD, $n = 3–8$. *Results are significantly different, $p < 0.05$; **results are significantly different, $p < 0.01$; ***results are significantly different, $p < 0.001$.

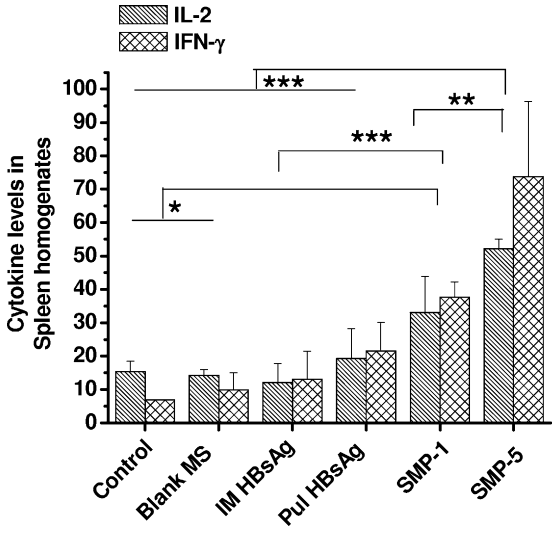

Fig. 7. Interferon-γ (IFN-γ) and interleukin-2 (IL-2) levels in spleen homogenates of rats immunized with different formulations and controls. IFN-γ and IL-2 levels were assayed 42 days after the first dose of the formulations. Data represent mean ± SD, $n = 3–5$. *Results are significantly different, $p < 0.05$; **results are significantly different, $p < 0.01$; ***results are significantly different, $p < 0.001$.

particles of HBsAg with a positive surface charge elicit enhanced humoral and mucosal immune responses and increased production of IL-2 and IFN-γ levels. However, more mechanistic studies are required to investigate the influence of PLGA particles of HBsAg in generation of cell-mediated immune response.

3.6. MTT cell viability studies

To evaluate the effect of unmodified PLGA (SMP-1) and surface-modified PLGA microspheres (SMP-5) on pulmonary epithelium, cell viability studies were conducted by MTT assay on Calu-3 cells. MTT, a tetrazolium salt, is cleaved by mitochondrial dehydrogenase in living cells to form a measurable, dark blue product called formazan. Damaged or dead cells display reduced dehydrogenase activity and therefore lower levels of formazan production compared to live cells. All the results are expressed as a percentage of the DMEM medium alone treatment group. As seen in Fig. 8, cell viability after saline treatment was high, ~90%, whereas treatment with the positive control, 0.1% SDS, caused a significant reduction in viability to ~16% ($p < 0.05$). Unmodified PLGA (SMP-1) and PEI-modified PLGA microspheres (SMP-5) showed concentration-dependent increases in cytotoxicity. However, compared to PEI alone, the SMP-5 formulation showed a reduction in toxicity levels. The reduction in cytotoxicity was to some extent due to the partial neutralization of PEI positive charges by negatively charged

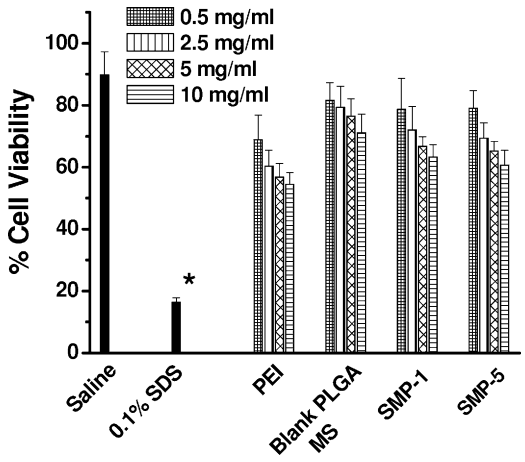

Fig. 8. Effects of unmodified PLGA microspheres (SMP-1) and PEI-modified PLGA microspheres (SMP-5) on the viability of Calu-3 cells. The test samples contained 0.5, 2.5, 5 and 10 mg/ml of different formulations. Data represent mean ± SD, $n = 8–16$. *Results are significantly different from other treatment groups, $p < 0.05$.

groups of PLGA as well as of the HBsAg antigen. This interpretation is supported by the zeta potential value of the SMP-5 formulation, which is only around +0.515 mV. Overall, the cytotoxicity produced by the SMP-5 formulation is comparable to those produced by the unmodified SMP-1 formulation and the blank PLGA microspheres.

On the whole, the data presented in this study suggest that the intrapulmonary administration of PLGA microspheres of HbsAg leads to generation of both humoral and mucosal immune responses. In addition, increased cytokine levels (IL-2 and IFN-γ) were also observed after pulmonary administration. In all three major mucosal tracts—oral, vaginal, and respiratory—an increase in secretory IgA levels was observed. Particles with a cationic surface charge were more efficacious in generating both humoral and mucosal immune response than were unmodified particles. However, since formulations tested were different in terms of their size, zeta potential, entrapment efficiency, and release profiles, it is reasonable to assume that in addition to surface charges, other factors such as particle diameter, drug content and hydrophibicity may play some roles in PLGA mediated generation of immune response. This study along with earlier published and unpublished studies (Thomas et al., 2008; Thomas, 2009) suggest that HBsAg administered via the pulmonary route produces both systemic and mucosal immune responses, and that this avenue of vaccination could be a viable alternative to currently available injectable recombinant hepatitis B vaccine.

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References

Amidi, M., Pellikaan, H.C., Hirschberg, H., de Boer, A.H., Crommelin, D.J., Hennink, W.E., Kersten, G., Jiskoot, W., 2007. Diphtheria toxoid-containing microparticulate powder formulations for pulmonary vaccination: preparation, characterization and evaluation in guinea pigs. Vaccine 25, 6818–6829.

Amorij, J.P., Saluja, V., Petersen, A.H., Hinrichs, W.L., Huckriede, A., Frijlink, H.W., 2007. Pulmonary delivery of an inulin-stabilized influenza subunit vaccine prepared by spray-freeze drying induces systemic, mucosal humoral as well as cell-mediated immune responses in BALB/c mice. Vaccine 25, 8707–8717.

Azzali, G., 2003. Structure, lymphatic vascularization and lymphocyte migration in mucosa-associated lymphoid tissue. Immunol. Rev. 195, 178–189.

Bivas-Benita, M., Ottenhoff, T.H., Junginger, H.E., Borchard, G., 2005. Pulmonary DNA vaccination: concepts, possibilities and perspectives. J. Control. Rel. 107, 1–29.

Brewer, J.M., Pollock, K.G., Tetley, L., Russell, D.G., 2004. Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles. J. Immunol. 173, 6143–6150.

Chisari, F.V., Ferrari, C., 1995. Hepatitis B virus immunopathogenesis. Annu. Rev. Immunol. 13, 29–60.

Constant, S.L., Bottomly, K., 1997. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. Annu. Rev. Immunol. 15, 297–322.

Cui, Z., Mumper, R.J., 2002. Intranasal administration of plasmid DNA-coated nanoparticles results in enhanced immune responses. J. Pharm. Pharmacol. 54, 1195–1203.

Debin, A., Kravtzoff, R., Santiago, J.V., Cazales, L., Sperandio, S., Melber, K., Janowicz, Z., Betbeder, D., Moynier, M., 2002. Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses. Vaccine 20, 2752–2763.

Edwards, D.A., Hanes, J., Caponetti, G., Hrkach, J., Ben-Jebria, A., Eskew, M.L., Mintzes, J., Deaver, D., Lotan, N., Langer, R., 1997. Large porous particles for pulmonary drug delivery. Science 276, 1868–1871.

Feng, L., Qi, X.R., Zhou, X.J., Maitani, Y., Wang, S.C., Jiang, Y., Nagai, T., 2006. Pharmaceutical and immunological evaluation of a single-dose hepatitis B vaccine using PLGA microspheres. J. Control. Rel. 112, 35–42.

Fifis, T., Gamvrellis, A., Crimeen-Irwin, B., Pietersz, G.A., Li, J., Mottram, P.L., McKenzie, I.F., Plebanski, M., 2004. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J. Immunol. 173, 3148–3154.

Gupta, P.N., Khatri, K., Goyal, A.K., Mishra, N., Vyas, S.P., 2007. M-cell targeted biodegradable PLGA nanoparticles for oral immunization against hepatitis B. J. Drug Target. 15, 701–713.

Gutierro, I., Hernandez, R.M., Igartua, M., Gascon, A.R., Pedraz, J.L., 2002. Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine 21, 67–77.

Jaganathan, K.S., Singh, P., Prabakaran, D., Mishra, V., Vyas, S.P., 2004. Development of a single-dose stabilized poly(D,L-lactic-co-glycolic acid) microspheres-based vaccine against hepatitis B. J. Pharm. Pharmacol. 56, 1243–1250.

Jaganathan, K.S., Vyas, S.P., 2006. Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally. Vaccine 24, 4201–4211.

Katare, Y.K., Muthukumaran, T., Panda, A.K., 2005. Influence of particle size, antigen load, dose and additional adjuvant on the immune response from antigen loaded PLA microparticles. Int. J. Pharm. 301, 149–160.

Lee, S.C., Oh, J.T., Jang, M.H., Chung, S.I., 1999. Quantitative analysis of polyvinyl alcohol on the surface of poly(D,L-lactide-co-glycolide) microparticles prepared by solvent evaporation method: effect of particle size and PVA concentration. J. Control. Rel. 59, 123–132.

Li, X., Kong, X., Shi, S., Zheng, X., Guo, G., Wei, Y., Qian, Z., 2008. Preparation of alginate coated chitosan microparticles for vaccine delivery. BMC Biotechnol. 8, 89.

Lu, D., Hickey, A.J., 2007. Pulmonary vaccine delivery. Expert Rev. Vaccines 6, 213–226.

Ma, R., Du, J.L., Huang, J., Wu, C.Y., 2007. Additive effects of CpG ODN and R-848 as adjuvants on augmenting immune responses to HBsAg vaccination. Biochem. Biophys. Res. Commun. 361, 537–542.

MacDonald, T.T., 2003. The mucosal immune system. Parasite Immunol. 25, 235–246.

McCluskie, M.J., Davis, H.L., 1999. Mucosal immunization with DNA vaccines. Microbes Infect./Institut Pasteur. 1, 685–698.

McDermott, M.R., Bienenstock, J., 1979. Evidence for a common mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory, and genital tissues. J. Immunol. 122, 1892–1898.

Mestecky, J., 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J. Clin. Immunol. 7, 265–276.

Nagler-Anderson, C., 2001. Man the barrier! Strategic defences in the intestinal mucosa. Nat. Rev. Immunol. 1, 59–67.

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.

Ogra, P.L., Faden, H., Welliver, R.C., 2001. Vaccination strategies for mucosal immune responses. Clin. Microbiol. Rev. 14, 430–445.

Pandit, S., Cevher, E., Zariwala, M.G., Somavarapu, S., Alpar, H.O., 2007. Enhancement of immune response of HBsAg loaded poly(L-lactic acid) microspheres against hepatitis B through incorporation of alum and chitosan. J. Microencapsul. 24, 539–552.

Patton, J.S., 1996. Mechanisms of macromolecules absorption by the lungs. Adv. Drug Deliv. Rev. 19, 3–36.

Patton, J.S., Fishburn, C.S., Weers, J.G., 2004. The lungs as a portal of entry for systemic drug delivery. Proc. Am. Thorac. Soc. 1, 338–344.

Rawat, A., Majumder, Q.H., Ahsan, F., 2008. Inhalable large porous microspheres of low molecular weight heparin: in vitro and in vivo evaluation. J. Control. Rel. 128, 224–232.

Rosenthal, K.L., Gallichan, W.S., 1997. Challenges for vaccination against sexually-transmitted diseases: induction and long-term maintenance of mucosal immune responses in the female genital tract. Semin. Immunol. 9, 303–314.
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., Briones, M., Ott, G., O'Hagan, D., 2000. Cationic microparticles: a potent delivery system for DNA vaccines. Proc. Natl. Acad. Sci. U.S.A. 97, 811–816.
Smith, D.J., Bot, S., Dellamary, L., Bot, A., 2003. Evaluation of novel aerosol formulations designed for mucosal vaccination against influenza virus. Vaccine 21, 2805–2812.
Thiele, L., Merkle, H.P., Walter, E., 2003. Phagocytosis and phagosomal fate of surface-modified microparticles in dendritic cells and macrophages. Pharm. Res. 20, 221–228.
Thomas, C., 2009. PhD Dissertation, Respirable particulate carriers for pulmonary delivery of hepatitis B vaccine. Texas Tech University Health Sciences Center, Texas USA.
Thomas, C., Rawat, A., Bai, S., Ahsan, F., 2008. Feasibility study of inhaled hepatitis B vaccine formulated with tetradecylmaltoside. J. Pharm. Sci. 97, 1213–1223.
Ungaro, F., De Rosa, G., Miro, A., Quaglia, F., La Rotonda, M.I., 2006. Cyclodextrins in the production of large porous particles: development of dry powders for the sustained release of insulin to the lungs. Eur. J. Pharm. Sci. 28, 423–432.
Vyas, S.P., Singh, R.P., Jain, S., Mishra, V., Mahor, S., Singh, P., Gupta, P.N., Rawat, A., Dubey, P., 2005. Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B. Int. J. Pharm. 296, 80–86.
Wee, S., Gombotz, W.R., 1998. Protein release from alginate matrices. Adv. Drug Deliv. Rev. 31, 267–285.
Wischke, C., Borchert, H.H., Zimmermann, J., Siebenbrodt, I., Lorenzen, D.R., 2006. Stable cationic microparticles for enhanced model antigen delivery to dendritic cells. J. Control. Rel. 114, 359–368.
Yang, Y.Y., Chung, T.S., Ng, N.P., 2001. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials 22, 231–241.