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Aerosolized PLA and PLGA Nanoparticles Enhance Humoral, Mucosal and Cytokine Responses to Hepatitis B Vaccine

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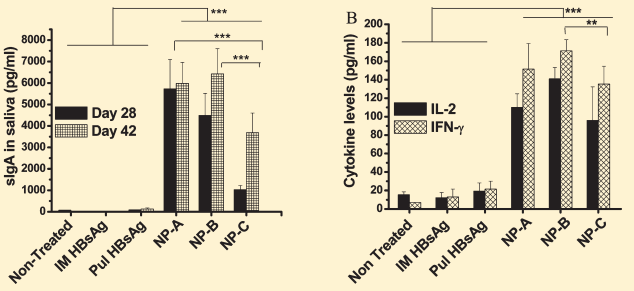
ABSTRACT: Porous poly(L-lactic acid) (PLA) and poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles were tested for pulmonary delivery of hepatitis B vaccine. In particular, the effects of particle size and hydrophobicity on mucosal and cell-mediated immune responses were investigated. Three formulations of PLA and PLGA nanoparticles containing a fixed amount of hepatitis B surface antigen (HBsAg) were prepared by a double-emulsion—solvent-evaporation method and characterized for surface morphology, charge, size, density and in vitro release. The immune responses were studied by measuring secretory IgA levels in mucosal fluids and quantitating cytokine levels in rat spleen homogenates. Particle uptake was studied in rat alveolar macrophages. Scanning electron microscopy revealed particles with smooth surfaces. Zeta potential measurements indicated that the particles carried negative surface charges. The antigen was continuously released for 42 days in phosphate buffer. Hydrophobic particles >500 nm elicited a more robust increase in secretary IgA, interleukin-2 and interferon-γ levels compared to hydrophilic particles <500 nm. Large hydrophobic particles were more efficiently internalized by rat alveolar macrophages compared to smaller hydrophilic particles. Calu-3 cell viability studies indicate that the viability of cells is not affected by nanoparticulate formulations. This study demonstrates that inhalable nanoparticles of HBsAg produce an enhancement of immune responses.



KEYWORDS: hepatitis B surface antigen, poly(D,L-lactic-*co*-glycolic acid), microspheres, pulmonary drug delivery, vaccine delivery, mucosal delivery

INTRODUCTION

Like many recombinant peptide and protein antigens, hepatitis B surface antigen (HBsAg) does not elicit a robust immune response when administered alone. Consequently, HBsAg is required to be administered with alum as adjuvants.[1,2] Despite the fact that alum adjuvanted hepatitis B vaccine has been used for more than two decades, the safety and compliance have negatively impacted the mass vaccination program against hepatitis B infection.[3] Indeed, needle-based administration of currently available thermolabile hepatitis B vaccine poses a major challenge for mass vaccination against hepatitis B in third world countries. The limitations of current hepatitis B vaccine have been documented in many publications and WHO reports.[4,5] Current intramuscular hepatitis B vaccine elicits only humoral antibody response. Mucosal vaccination via the pulmonary route would induce both mucosal and systemic immune response. In the case of hepatitis B vaccination, a mucosal vaccine would provide more robust immune response because the disease is transmitted via the mucous membrane.[6,7] Secretory immunoglobulin antibodies (sIgA) produced upon mucosal administration can prevent the entry of pathogens through the mucosal sites. For these reasons, various approaches to develop and deliver hepatitis B vaccine via the mucosal route have been an intensive area of research for many years now. The strategies that have been tested include use of polymeric carriers such as chitosan, poly(D,L-lactide-*co*-glycolide) (PLGA), and poly(lactic acid) (PLA) as adjuvants and/or delivery carrier.[1,8] Indeed, both micro- and nanoparticles made of these polymers have been used to develop vaccines against many infectious diseases.[1,2,8]

However, to achieve a predictable and strong immune response upon administration of particulate vaccines via the mucosal or parenteral routes, it is imperative to optimize the carriers for optimal size, surface charge and hydrophobicity. In fact, the factors that may influence immune response produced by polymeric particles include inner structure of particulate carriers, use of adjuvants, surface charge, particle size, and surface hydrophobicity.[9,10] These parameters can also affect differential uptake by macrophages and dendritic cells, which play an important role in

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generating the immune response.[11,12] For example, particles prepared with hydrophobic and higher molecular weight polymers tend to be phagocytosed to a greater extent compared to their hydrophilic counterparts.[12−15] Polymeric particles of poly(ε-caprolactone), which is more hydrophobic than PLGA, are taken up more efficiently by Caco-2 cells and produce a higher level of antibody in response to diptheria toxoid.[16] Interestingly, PLGA polymers with a hydrophilic surface are also reported to be readily taken up by the dendritic cells.[14] Similar to hydrophobic or hydrophilic characters of particles, surface charge may influence the immune responses generated by nanoparticles. Cationic nanoparticles are reported to be more efficient in generation of antibody and CD8 T cell responses compared to neutral or anionic particles.[12,17,18] However, there are conflicting reports regarding the influence of particle size on the generation of immune responses. Some studies suggest that particles with a size range of about 5 μm are more efficacious in generating immune response upon mucosal administration, while others showed that particles in the size range of ∼1 μm are more efficiently taken up by antigen presenting cells than 500 and 200 nm size particles and subsequently produce a stronger immune response.[9] Contrasting these observations, others demonstrated that particles of ∼500 nm size are more suitable for uptake than particles of 1−5 μm size.[19,20] Immune response may also vary depending on the route of administration because of the involvement different sets of APCs at different routes of administration.[21,22] Indeed, a combination of factors are involved that may modulate immune response produced by particulate carrier based vaccination.

However, most of the previously published reports investigated the influence of the above-mentioned parameters of particulate carriers in generating immune response upon nasal, oral and parenteral vaccination.[9,21,23−25] Little is known about the influence of surface charge, particle size and hydrophobicity in the generation of mucosal immune response upon pulmonary administration of polymeric particle-based vaccines. Recently, we have shown that PLGA microparticles (∼5 μm) of HBsAg show size dependent increase in immune response after pulmonary administration.[26] We have also shown that the presence of positive surface charge on HBsAg containing microparticle leads to significant enhancement in mucosal and cell mediated immune response.[27] In addition to microparticles, nanoparticles made of different polymers have also been used for delivery of hepatitis B surface antigen.[23,25,28−30] It has been suggested that compared to micrometer size particles, polymeric nanoparticles are more efficacious in generating immune response[31] because nanoparticles offer greater surface area for adsorption that allows a higher antigen:polymer ratio and for this reason nanoparticles are taken up more efficiently than microparticles.[17,31,32] However, there is no study concerning the use of nanoparticles for pulmonary delivery of hepatitis B vaccine. It is still unknown how the size and hydrophobicity of nanoparticulate formulations of HBsAg may affect mucosal and cell-mediated immune response. In this study, we seek to use nanoparticles as carrier of pulmonary vaccination of hepatitis B vaccine and investigate the influence of size and hydrophobicity in generating mucosal and cell mediated immune response.

MATERIALS AND METHODS

Materials. Poly(D,L-lactide) (PLA) (inherent viscosity 0.55−0.75 dL/g; average molecular weight ∼93 kDa), and poly(D,L-lactide-*co*-glycolide) polymer (PLGA-50:50: inherent viscosity 0.55−0.75 dL/g, average molecular weight =52.4 kDa; PLGA-85:15, inherent viscosity 0.55−0.75 dL/g, average molecular weight = 85.2 kDa) were purchased from Boehringer Ingelheim (Lactel Absorbable Polymers, Pelham, AL). Hepatitis B surface antigen (HBsAg) in phosphate buffer at a concentration of 2 mg/mL was provided as a gift by Shantha Biotechnics (Hyderabad, India). Poly(vinyl alcohol) (PVA) was purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO). The BCA and micro BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL). Fluorescein isothiocyanate-conjugated bovine serum albumin (BSA−FITC) and tetramethylrhodamine isothiocyanate−phalloidin (TRITC−phalloidin) were purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO). DRAQ5 (Biostatus Ltd., Leicestershire, U.K.) was provided by Dr. Ulrich Bickel from Texas Tech University Health Sciences Centre, Amarillo, TX.

Formulation and Characterization PLA and PLGA Nanoparticulate Vaccines. Three sets of nanoparticulate formulations were prepared using PLA or PLGA by the w/o/w double emulsion solvent evaporation method. Polymeric nanoparticles prepared with PLA, PLGA 85/15 and PLGA 50/50 were designated as (i) NP-A, (ii) NP-B and (iii) NP-C, respectively. Briefly, 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 0.250 g of PLA or PLGA polymer with a probe sonifier (Branson Sonifier 450, Branson Ultrasonics Corporation, Danbury, CT) for 60 s in an iced bath. The resulting water-in-oil (w/o) primary emulsion was then poured into 25 mL of 5.0% w/v polyvinyl alcohol (PVA) aqueous solution (external aqueous phase, EAP) and emulsified by homogenization for 30 min at 21,500 rpm. The secondary emulsion was then stirred overnight at room temperature for evaporation of organic phase. The resulting polymeric particles were washed thrice and lyophilized to get free-flowing nanoparticles. A set of blank nanoparticles without HBsAg was also prepared. For rat alveolar macrophage uptake studies, fluorescein isothiocyanate-conjugated bovine serum albumin (BSA−FITC) was encapsulated in PLGA nanoparticles. Fluorescent particles of two different size ranges were prepared by varying the energy input during the primary emulsification as well as varying the homogenization speed during the secondary emulsification. The average size of one set of particle sizes was close to 1−1.5 μm (NP-F-1), and that for another set of particles was around 711 nm (NP-F-2).

Physical Characterization of Nanoparticles. The nanoparticulate formulations were characterized for surface morphology, particle size and zeta potential. *The morphology* of the formulations was studied by scanning electron microscopy (SEM). The samples for SEM were prepared by sprinkling a small amount of powdered and freeze-dried formulation (1−2 mg) onto a double sided adhesive tape attached to an aluminum stub and then sputter-coated with gold under argon (Emitech K550X, Kent, U.K.). The formulations were then examined by SEM and photomicrographs were taken by using a Hitachi S-3400N (Freehold, NJ) scanning electron microscope.

Particle size distribution and mean volume based diameter of the formulations was studied by dynamic light scattering using Nanotrac Ultra from Microtrac (North Largo, FL) particle size analyzer. Samples for particle size analysis were prepared by dispersing the freeze-dried formulations in 0.2% w/v solution of Tween 80. The experiments for particle sizing were performed in triplicate, and data is presented as mean ± SD.

The tapped density of the nanoparticles was estimated as described previously.[33] An aliquot of 100 mg of particles was transferred to a 10 (±0.05) mL graduated cylinder and initial volume was recorded. Tapped density of the formulations (ρ) was calculated as the ratio between sample weight (g) and the volume (mL) occupied after 200 tappings. *The zeta potential* measurements of nanoparticles were measured using a NICOMP 380 ZLS instrument (PSS NICOMP, Santa Barbara, CA). For zeta potential measurements, HBsAg entrapped formulations were dispersed in deionized water containing 1 mM potassium chloride solution as previously reported.[9] Each sample was tested in triplicate and data reported as ± SD.

Entrapment Efficiency. The amount of the antigen loaded in PLA and PLGA nanoparticles was estimated by the micro BCA method after modifying a previously published technique.[34] This commercial assay kit is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein (Pierce, Rockford, IL). The detection limit of the micro BCA method is 0.5−20 μg/mL. Further, possible degradation products of PLA/PLGA particles, lactic and glycolic acid, are unlikely to interfere with the assay because micro BCA assay reagents only interact with chemicals with reducing potential, chelating agents, and strong acids or bases. To conduct this assay, 5 mg of HBsAg entrapped nanoparticles was dissolved in 1 mL of 5% w/v sodium dodecylsulfate (SDS) in 0.1 M NaOH solution. NaOH dissolves the polymers and help release of the protein and SDS serve as solubulizer of the protein. SDS does not interfere with micro BCA reagents, and it has been extensively used at concentrations between 1 and 5% to determine entrapment efficiency of PLA/PLGA particles of peptides and small molecular weight drugs.[35−37] The dispersion was then vortexed and centrifuged at 12,000 rpm at 4 °C for 10 min to remove any undissolved PLA. The supernatant was removed carefully and HBsAg antigen content in the supernatant was determined using the micro BCA method. Blank nanoparticles without HBsAg containing only sucrose and $Mg(OH)_2$ were used as control. The entrapment efficiency of the formulations was expressed as the percentage of calculated antigen loaded in the nanoparticles with the actual amount of antigen added during the preparation of the nanoparticles.[33]

In Vitro Release Experiments. The in vitro release studies were performed in phosphate buffer. An aliquot (10 mg) of freeze-dried nanoparticles of HBsAg was suspended in microcentrifuge 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). At predetermined time points, beginning at time zero, the vials were removed from the incubator and centrifuged at 4000 rpm for 10 min at 4 °C. The samples were collected for a period of 42 days. About 400 μL of the supernatant was collected and fresh PBS was added to the vial in order to maintain the pH. The antigen concentrations in the supernatants were determined by micro BCA assay. The release profile of HBsAg was calculated by taking into account the entrapment efficiency of the HBsAg-loaded nanoparticles for each formulation and was reported in terms of cumulative antigen released versus time. Furthermore, the amount of antigen released (percent 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.[33] All the samples were tested in triplicate, and the data are expressed as mean ± SD.

Pulmonary Immunization Studies. Pulmonary immunization studies were performed according to our previously published method.[38] Female Sprague−Dawley (Charles River Laboratories, Charlotte, NC) rats weighing between 150 and 200 g were used for all the immunization studies. Prior to the experiment, rats were anesthetized by an intramuscular injection of an anesthetic cocktail containing xylazine (20 mg/mL) and ketamine (100 mg/mL) and animals were divided into five groups (8−12 rats in each group) to receive the following treatments: NP-A, NP-B and NP-C formulations, plain HBsAg administered via the pulmonary route, and intramuscular injection of HBsAg. The dose of HBsAg administered was 10 μg/animal, 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., Wyndmoor, PA) as described previously.[26,38] The microsprayer generates aerosols through a patented atomizer at the tip of the device. The atomizer has a diameter of 1.5 mm and produces an aerosol plume of 25−30 μm size.

Blood Sample Collection and Evaluation of Serum Immune Response. The humoral immune response was evaluated by determining serum IgG levels. Blood samples were collected on days 7, 14, and 28 from rats that received test and control formulations via the pulmonary or intramuscular route. Briefly, the animals were anesthetized as described above and blood was collected from the tail vein. The collected samples were centrifuged at 6000 rpm for 10 min in a microcentrifuge 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 quantitative ELISA kit (HBsAb, International Immuno Diagnostics, Foster City, CA) as per published procedure and manufacturers' protocol.[26,38] Briefly, the microplates for ELISA were precoated with purified heat-inactivated HBsAg. The samples and the standards were added into the microplate and incubated for 60 min at 37 °C. Following incubation, microplates were washed; the enzyme conjugate was added into the wells and again incubated for 60 min at 37 °C. The reaction was stopped by adding sulfuric acid, and the absorbance was measured in a microplate reader at 450 nm. The kit was standardized in our laboratory to further confirm the limit of quantitation using the standard samples provided in the kit.

Evaluation of Mucosal Immune Response. To study the mucosal immune response, secretory antibody levels (sIgA) in salivary, vaginal 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.[24,39] For collection of salivary secretion, an approximately 10 mg/mL sterile solution of pilocarpine was administered intraperitoneally (0.1−0.2 mL, depending on the weight of the rat). Within a few minutes of injection, the rats began to salivate and the saliva was collected using a pipet. 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 pipet. The solution was withdrawn and again 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 as described previously.[38] In brief, the respiratory apparatus was exposed by a midlevel 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, reinstilled 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 were determined by using a commercially available ELISA kit (Bethyl Laboratories, Montgomery, TX). The ELISA kit used for estimation of sIgA levels had a quantification range between 15.625 and 1000 pg/mL. The kit was standardized in our laboratory to further confirm the limit of detection. To minimize interference, the samples were diluted up to 50 times with the diluting reagent provided in the kit.

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.[40] 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 were 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 and published procedure.[27] For estimation of both IL-2 and IFN-γ, standards provided by the manufacturer were used to determine the concentrations of the test samples. First, 96-well microplates were coated with the capture antibody. Following a cycle of incubation and washing, aliquots of standards and test samples were added to the wells. The detection antibody and streptavidin-HRP was then added to each well and incubated for 20 min for color development. The reaction was then stopped by adding 2 N H_2SO_4 and the optical density (OD) was measured at 450 nm.

Particle Uptake by Rat Alveolar Macrophages. To conduct uptake study by rat alveolar macrophages, we first collected alveolar macrophages from male Sprague−Dawley (Charles River Laboratories, Charlotte, NC) rats weighing between 300 and 350 g after modifying the bronchoalveolar lavage method reported by us and other groups.[26,38,41] The lungs were isolated from anesthetized animals, and lavage was performed by instilling 5 mL of calcium and magnesium free Dulbecco's phosphate buffered saline (DPBS) (Gibco, Grand Island, NY) containing 0.5 mM disodium EDTA (Fisher Scientific, Fair Lawn, NJ). The procedure was repeated until a volume of around 30 mL was obtained. The lavaged fluid was centrifuged at around 400*g* for 10 min to obtain pellets of alveolar macrophages. The cell pellet was resuspended in 500 μL of 0.1% BSA (w/v) in Hanks balanced salt solution (phenol red free, pH 7.4, Gibco, Grand Island, NY) and counted in a hemocytometer. A cell density of 4×10^5 cells per mL was added on a coverslip (12 mm in diameter) placed in a 24 well cell plate. The plate was then incubated for 1 h in a humidified chamber at 37 °C in 5% CO_2. Following an hour of incubation, the media containing the non-adherent cells were aspirated and the plates were washed with PBS.

For uptake study, an aliquot of PLGA particles dissolved in medium at a concentration of 1 mg/mL was added into a 24 well plate and incubated for 1 h in a humidified chamber at 37 °C in 5% CO_2. The cells were then washed with PBS and fixed with 3% freshly prepared paraformaldehyde for 15 min at room temperature. The cells were washed again with PBS to remove the paraformaldehyde and permeabilized with 0.2% Triton X-100 for 40 min at room temperature. Following permeabilization, tetramethylrhodamine isothiocyanate−phalloidin (TRITC−phalloidin) was added onto the coverslip to stain the actin cytoskeleton microfilament network of the macrophages and incubated at room temperature in the dark for 90 min. After the incubation period, the cells were washed three times with PBS. Nuclear staining was performed with DRAQ5 by incubating for 10 min by following the manufacturer's protocol, after which the cells were again washed three to four times. The glass coverslips were then carefully removed, placed on a glass slide by using a drop of antifade solution, and sealed. Uptake of the fluorescent particles by the alveolar macrophages was viewed under a Leica confocal microscope (TCS SL; Leica Microsystems, Heidelberg, GmbH) with 63×/1.4 oil objective and immersion oil. FITC−BSA was excited with the 488 nm line of the system's argon laser, and TRITC−phalloidin was excited with the 546 nm line of the HeNe laser whereas the nuclear staining with DRAQ5 was visualized by excitation at 633 nm. Leica confocal software, version 2.61 Build 1537, was used for image acquisition and analysis.

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 three formulations was studied: NP-A, NP-B and NP-C. The concentrations of PLGA particles used were 0.5 mg/mL, 2.5 mg/mL, 5 mg/mL and 10 mg/mL. Saline and sodium dodecyl sulfate (SDS, 0.1%) were used as negative and positive controls, respectively. The cells were plated into 96-well microtiter 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. The cells were plated into 96-well microtiter 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.[33] 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 microtiter-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.

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.

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 comparisons by GraphPad InStat software (GraphPad Software, San Diego, CA). *P*-values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Characterization of PLA and PLGA Nanoparticles. The surface morphology of the PLA and PLGA nanoparticles loaded with HBsAg were studied using a scanning electron microscope (SEM). PLA-based particles (NP-A) were almost perfectly spherical in shape with a uniform distribution of size (Figure 1A). Like

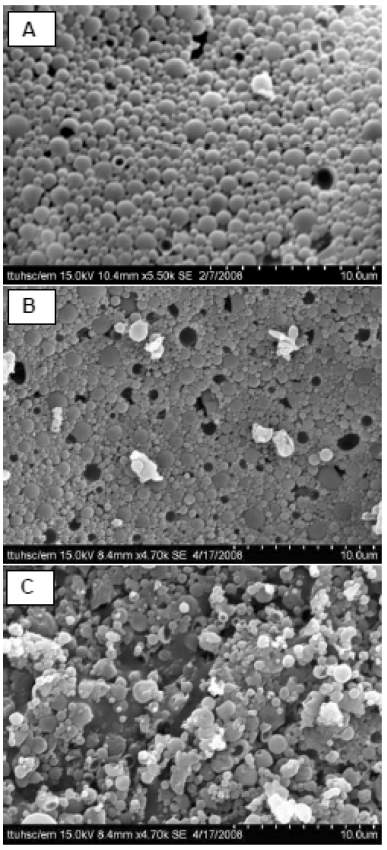

Figure 1. Surface morphology of HBsAg-loaded nanoparticles prepared with PLA and PLGA: (A) NP-A, (B) NP-B, and (C) NP-C.

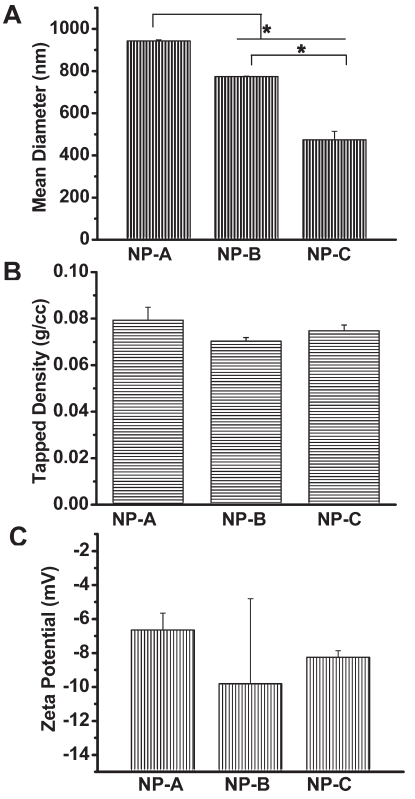

Figure 2. Physical characterization of HBsAg-loaded nanoparticles: (A) mean diameter, (B) tapped density, and (C) zeta potential. Data represent mean ± SD (n = 3) (*results are significantly different, $p < 0.001$).

PLA-based formulations, particles prepared with PLGA 85/15 (NP-B) were spherical in shape and had smoother surface. However, particles prepared with PLGA 50/50 (NP-C) showed rather rough surface and agglomerates of particles (Figures 1B and 1C). Particles' varying surface morphology can be explained by differences in hydrophilicity of polymer. A hydrophilic polymer matrix tends to retain more water; upon drying, polymeric film around the particles may shrink, which eventually leads to the development of rough surfaces. Further, hydrophilic polymers facilitate formation of particle−particle contact points that may result in aggregation or agglomeration.[33] The size of nanoparticles prepared with PLA, PLGA 85/15 and PLGA 50/50 were ∼940, 774, and 474 nm, respectively, suggesting that the particle size decreased with the decrease in the molecular weight of the polymers (Figure 2A). The variation in the size of particles made of different polymers can be explained by their differences in their molecular weight and the amount of surfactant added during the emulsification. The molecular weight of PLA is higher than that of the other two polymers, and the high molecular weight of the polymer is reported to be associated with the increase in size of the particles.[42−44] In fact, the molecular weight of polymers used in this study can be ranked as follows: PLA > PLGA 85:15 > PLGA 50:50, which correlates well with the particle size data presented in Figure 2A. The use of stabilizing surfactant, PVA, during the emulsification process may have also contributed to the differences in particle size.[42−45]

The tapped density data presented in Figure 2B show that all particles are highly porous in nature because the porosity of the formulation is inversely proportional to the density of the formulation. The tap density of the three formulations was between 0.0703 and 0.0793 g/mL with the PLA based NP-A formulation showing the lowest porosity (Figure 2B). No significant differences in the porosity of three formulations were observed because the volume of the IAP was the same for all particles ($p > 0.05$). The zeta potential was negative for all the three preparations with values ranging from −6.66 to −9.82 mV (Figure 2C), suggesting that the particles carried negative surface charge, which is in agreement with published studies.[9,24,46]

Entrapment Efficiency. The entrapment efficiency of HBsAg antigen loaded into the PLGA nanoparticles was determined by using the micro BCA protein assay kit. The entrapment efficiency of the nanoparticles was between 80 and 93% (Figure 3A). The entrapment efficiency of the PLA based nanoparticles (NP-A) was higher than that of PLGA based particles (NP-B and NP-C), although the entrapment efficiencies of the two PLGA based particles were very similar with a value of 81% and 80% for the NP-B and NP-C particles, respectively. Importantly, the entrapment efficiency of the particles presented in this study is significantly higher than that of previously reported PLGA/PLA particles of HBsAg. The differences between the entrapment efficiencies of previously reported HBsAg-PLGA/PLA nanoparticles and those reported in this manuscript may stem from two factors: First, use of protein stabilizers and cryoprotectant may have contributed to enhanced entrapment efficiency by increasing the stability of primary emulsion as well as protecting the antigen from degradation. This reasoning agrees with a previous report that suggests that the use of sucrose in the internal phase increases the entrapment efficiency.[47] Further, $Mg(OH)_2$ was used to prevent acid induced degradation of protein. Lactic and glycolic acids released due to

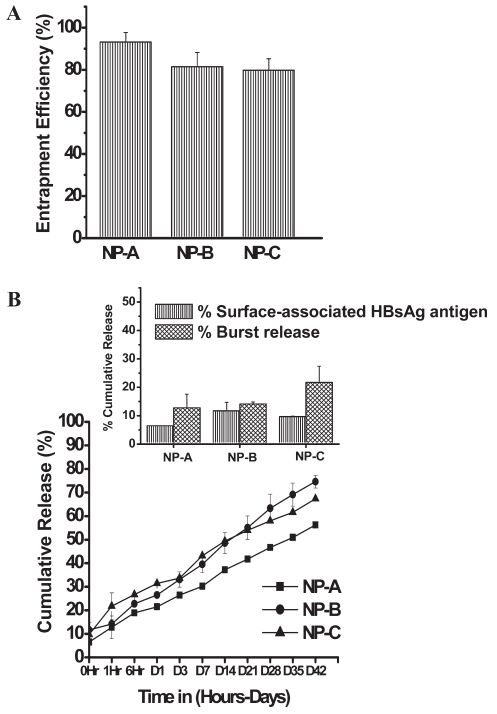

Figure 3. (A) Entrapment efficiency and (B) in vitro release profiles of HBsAg-loaded PLA and PLGA nanoparticles (% cumulative release profiles for 42 days). Inset shows % burst and surface-associated HBsAg release; data represent mean ± SD (n = 3).

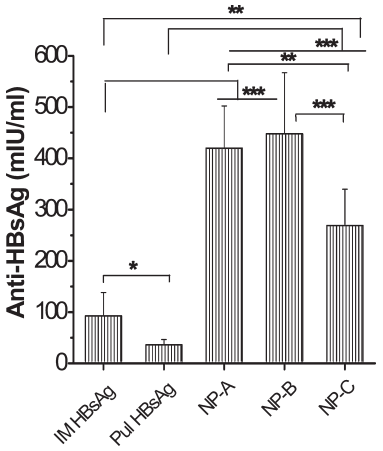

Figure 4. Immune response profiles of nanoparticulate based formulations at the end of 28 days after dosing of NP-A (PLA based, 943 nm); NP-B (PLGA 85:15 based, 774 nm) and NP-C (PLGA 50:50 based, 474 nm) formulations on days 0 and 14. Data represent mean ± SD, n = 6−9 (*results are significantly different, $p < 0.05$; **results are significantly different, $p < 0.01$; ***results are significantly different, $p < 0.001$).

hydrolysis of PLGA cause a drop in pH in the microenvironment of particles. $Mg(OH)_2$ prevents any such drop in pH and subsequently helps stabilize protein against acid-induced degradation. As $Mg(OH)_2$ was suspended in the internal aqueous medium, it is likely to be part of the core of the particles and function as stabilizer. It is not going to leach out from the internal phase to the external oil phase, methylene chloride, in which $Mg(OH)_2$ is insoluble. Similar to this study, $Mg(OH)_2$ has been used in internal aqueous phase to prevent acid induced degradation of protein.[35] Second, a higher concentration of PVA in the external phase has further led to an increase in entrapment efficiency by increasing the stability of secondary emulsion and preventing diffusion of HBsAg from the internal aqueous phase to the external phase.[48,49]

In Vitro Release Profiles. The cumulative release profiles of HBsAg loaded PLA and PLGA nanoparticles were studied for a period of 42 days (Figure 3B). The release of HBsAg from the nanoparticles was mainly influenced by the lactide to glycolide ratios of the polymers.[50,51] PLA-based particles showed the least surface-associated (6.45%) and burst release (12.80%) of the antigen (Figure 3B). On the other hand, PLGA 50/50 particles showed more surface-associated and burst release of the antigen compared to PLGA 85/15 particles. For example, NP-B nanoparticles released 14% of the antigen in the burst phase, while 22% antigen was released for NP-C particles. NP-A formulation showed almost a constant release of the antigen throughout the release study period. PLA nanoparticles (NP-A) showed a cumulative release of 56% at the end of the 42 day study, while NP-B and NP-C showed a cumulative release of around 64% and 78%, respectively. Differences in the ratios of glycolic to lactic acid in the polymers used to prepare the formulations can explain the differences in the release profiles. There was a higher proportion of glycolic acid in PLGA 50:50 compared to either plain PLA (having no glycolide) or PLGA 85:15. Increase in the glycolide content increases the hydrophilicity of PLGA, which in turn increases the hydration of the polymer and contributes to the increase in the release of the antigen.[52,53] Moreover, for PLGA nanoparticles, the differences in the size of the nanoparticles may also contribute to the total antigen release from the polymeric material.[54]

Estimation of Serum and Mucosal Antibody Titers from Different Sized Nanoparticles. When the total anti-HBsAg antibody levels were measured using an ELISA kit, similar to the results obtained in our previously published studies,[26,27] when plain HBsAg was administered by intramuscular (im) injection, it showed an antibody profile that was significantly higher compared to the plain HBsAg administered by the pulmonary route (Figure 4, $p < 0.05$).

However, the HBsAg administered by the im route showed a decline in the immune response after 21 days, which could be due to the deviation from the standard immunization schedule. Unlike the plain im or pulmonary HBsAg administration, the different sized PLA and PLGA nanoparticulate formulations showed a significant increase in the immune response after 28 days (Figure 4). The highest increase in serum anti-HBsAg levels was produced by NP-B formulation, followed by NP-A and NP-C formulations. The NP-A and NP-B formulations did not show any significant differences between them after 28 days of the immunization studies ($p > 0.05$). However, when the two formulations were compared with NP-C formulations, both PLA based NP-A nanoparticles ($p < 0.01$) and the PLGA 85:15 based NP-B formulations ($p < 0.001$) showed statistically significant levels of immune response after pulmonary immunization. The immune response data (Figure 4) shows clearly that antigen encapsulated in the nanoparticles was biologically active and was capable of generating antibody specific to HBsAg much higher than the required minimal clinically protective level, >10 mIU/mL as reported earlier.[40,55]

The mucosal immune responses were determined by measuring sIgA levels in salivary secretions and the vaginal wash on days 28 and 42 and in the BAL fluid on day 42. The sIgA response produced in saliva after im administration of plain HBsAg was negligible (Figure 5A). When plain HBsAg was administered by the pulmonary route, it led to a slight increase in sIgA levels compared to both

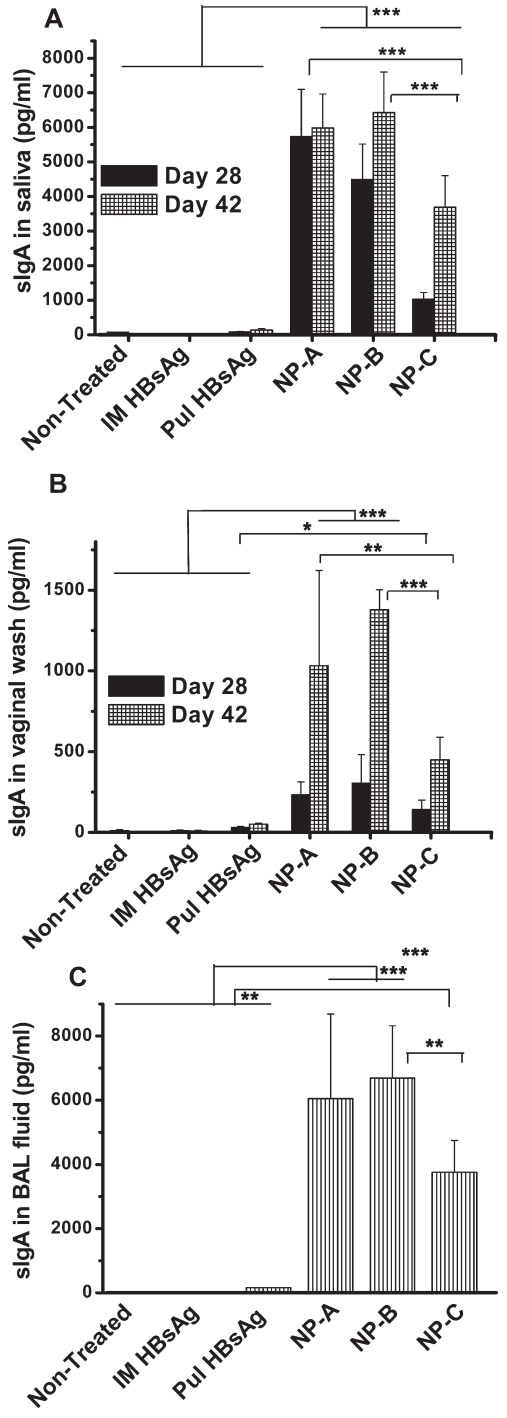

Figure 5. Mucosal immune response profiles after 2 doses of vaccine or control formulation on days 0 and 14. Secretory IgA (sIgA) levels in (A) salivary secretion, (B) vaginal wash, and (C) bronchoalveolar lavage (BAL) fluid of rats immunized with different formulations, and control groups. Data represent mean ± SD, $n = 3-8$ (*results are significantly different, $p < 0.05$; **results are significantly different, $p < 0.01$; ***results are significantly different, $p < 0.001$).

the untreated animals and im vaccine. However, when nanoparticulate HBsAg formulations were administered by the pulmonary route, all three formulations showed a statistically significant increase in sIgA levels compared to three control groups: untreated, im and animals treated with plain inhaled HBsAg ($p < 0.001$). Similar to the serum antibody levels, the highest increase in sIgA levels was produced by NP-B formulation, followed by NP-A and NP-C formulations. However, the differences between sIgA levels for NP-A and NP-B formulations were not statistically significant (Figure 5A). Similarly, all three formulations produced increase in sIgA levels in vaginal washes. But NP-A and NP-B formulations showed a higher statistically significant increase in sIgA levels in the vaginal wash (Figure 5B) compared to three control groups ($p < 0.001$) and ($p < 0.05$) NP-C formulation. In the case of sIgA levels in BAL, all three groups also showed statistically significant difference compared to the control groups with NP-A and NP-B showing $p < 0.001$ while NP-C showed a significance level of $p < 0.01$ (Figure 5C). Both the serum and mucosal immune response data can be explained on the basis of particle size of the formulations and hydrophobicity, i.e., lactide to glycolide ratio of the polymer used in the preparation of the nanoparticles. Based on the particle size analysis, the three formulations can be ranked as NP-A > NP-B > NP-C. NP-A formulations showed a particle size in the range of 940 nm which is very close to micrometer range; the particle size of NP-B was around 774 nm, and it was around 474 nm for NP-C formulations (Figure 2A). NP-A and NP-B formulation particle sizes are more close to each other and they have shown similar increase in antibody levels as compared to NP-C formulations. Further, NP-C formulation is more hydrophilic compared to NP-A and NP-B formulation and so the reduced hydrophilicity may have led to decreased immune responses. Previously, it has been shown that the differences in immune responses may stem from the differences in particle size: Intranasal administration of 1000 nm size particles showed higher serum IgG responses compared to the 500 or 200 nm size particles.[9] As discussed below in the macrophage uptake study, depending on the particle size, particles may be preferentially taken up by either dendritic cells or macrophages. As these formulations were of nanometer size, they are likely to increase antibody levels probably because of preferential uptake by dendritic cells.[11,12,56,57] Further, the differences in hydrophobicities of particles can also make a difference in the uptake because more hydrophobic particles have a propensity to adsorb more to the cell surface compared to its hydrophilic counterpart perhaps because of enhanced nonspecific interaction with the hydrophobic cell surface.[58]

Estimation of Cytokine Levels. Cytokine levels in spleen homogenates were measured 6 weeks following the receipt of first dose of the formulations. It is important to point out that although sIgA levels were evaluated on days 0, 28 and 42, cytokine levels were determined at the end of 42-day treatment period when cytokine level is expected to be much higher compared to the earlier time point on day 28. The rationale for this experimental design was to reduce the number of animals and to measure cytokine level when it reaches its maximal level. As shown in Figure 6, compared to three control groups—no treatment, im injection of hepatitis B and plain HBsAg administered by pulmonary route—all three nanoparticulates showed a significant increase in both IL-2 and IFN-γ levels at the end of 6 weeks (Figure 6, $p < 0.001$).

The cytokine levels produced by NP-B particles were slightly higher than than the NP-A particles, but the differences were not statistically significant ($p > 0.05$). However, NP-B formulation showed statistically significantly enhancement in the both the cytokine levels compared to NP-C nanoparticles ($p < 0.01$). These differences can be attributed to the differences in the particle size and hydrophobicity of the nanoparticles. In fact, compared to our previous studies with microparticles of 5 μm size,[27] nanoparticles used in this study produced more pronounced increased in

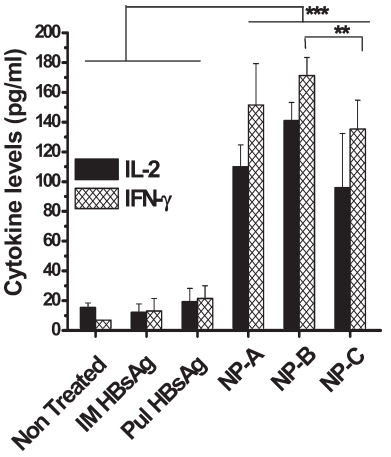

Figure 6. Interferon-γ (IFN-γ) and interleukin-2 (IL-2) levels in spleen homogenates of rats immunized with different formulations, and control groups. IFN-γ and IL-2 levels were assayed 42 days after the first dose of the formulations. Data represent mean ± SD, n = 3−8 (*results are significantly different, $p < 0.05$; **results are significantly different, $p < 0.01$; ***results are significantly different, $p < 0.001$).

endogenous cytokine levels. This data also agrees with studies published by Kanchan et al.,[59] who reported that nanoparticles are more preferentially taken up by the macrophages (200−600 nm) compared to the microparticles (2−8 μm). Similar increases in cellular responses were observed for nanoparticles in the size of 300 nm after subcutaneous administration of PLGA based nanoparticles.[30] Overall, this set of studies indicates that particles with a size range between 700 and 1000 nm are ideal for generation of robust humoral, mucosal and increased levels of cytokines after pulmonary vaccination.

Particle Uptake Studies by Rat Alveolar Macrophages. In this set of experiments, we have studied the influence of particle size on the uptake by alveolar macrophages, an antigen presenting cell. This study was also designed to indirectly rule in or rule out if the uptake by dendritic cells has any contribution to generation of immune response. For this study, we used particles of two different size ranges: (i) NP-F-1 with a diameter of ∼1−1.5 μm and (ii) NP-F-2 with a diameter of NP-F-2 ∼700−750 nm. These two size ranges for fluorescent particles were selected based on the size of particles used in immunization studies. In fact, NP-A and NP-B nanoparticles used in mucosal immunization studies fall within the size range of of NP-F-1 and NP-F-2 formulations, respectively, used in this study. The confocal images presented in Figures 7A and 7B suggest that larger particles (NP-F-1: 1−1.5 μm) showed more preferential uptake compared to smaller particles (NP-F2: 711 nm). The reduced uptake by macrophages could be because other main antigen presenting cell, dendritic cells, may be contributing to the generation of enhanced mucosal immunity and cellular responses as reported earlier.[11,12,56,57] Previously, it has been shown that dendritic cells show greater of uptake of particles in the range of 500 nm or below and the uptake may be also influenced by other factors such as surface charge and hydrophobicity of the polymer used in the formulation.[9,10,50,51,54] In fact, particles that resemble the size of virus (20−200 nm) are preferentially taken up by dendritic cells, while particles that are close to the size of bacteria (0.5 to 5 μm) are more efficiently taken up by macrophages.[18] Further, we have shown that PLGA microparticles show size dependent uptake by rat alveolar macrophages: particles greater than 10 μm either showed no uptake by the

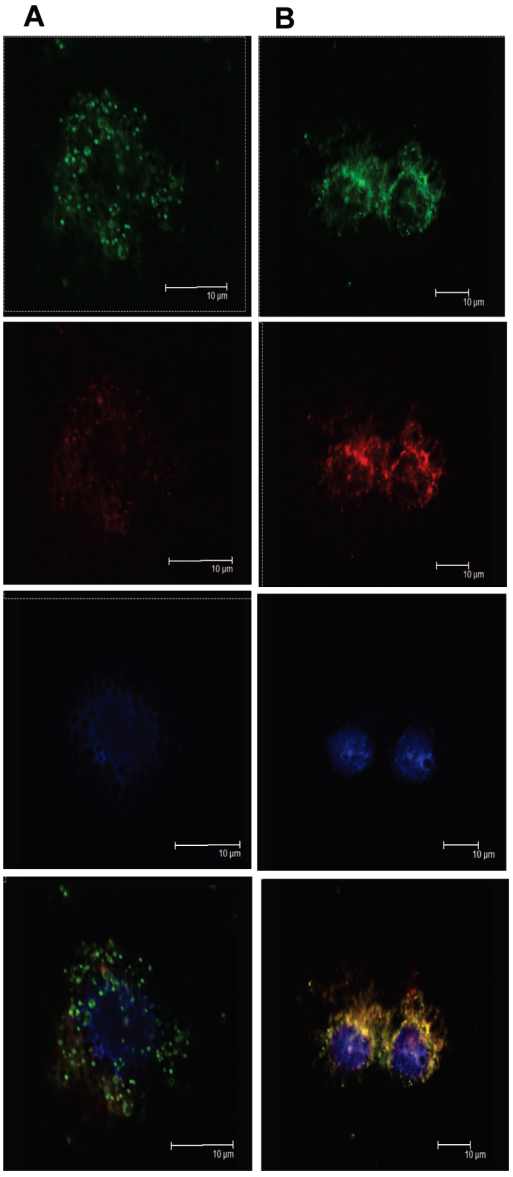

Figure 7. CLSM images of rat alveolar macrophage uptake study: (A) BSA−FITC PLGA particles of ∼1−1.5 μm size showing increased uptake by rat alveolar macrophages; (B) BSA−FITC nanoparticles of 700−750 nm size showing lesser uptake at the end of 1 h incubation. The actin cytoskeleton was visualized by labeling with rhodamine−phalloidine (red), the microspheres by labeling with FITC (green), and far red fluorescent DNA dye DRAQ5 was used as a nuclear stain.

macrophages or surface attachment and microparticles in the range of 4−5 μm showed enhanced uptake.[26]

MTT Cell Viability Studies. To evaluate the effect of nanoparticulate formulations 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 Figure 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% ($p < 0.05$). Each of the three nanoparticulate formulations,

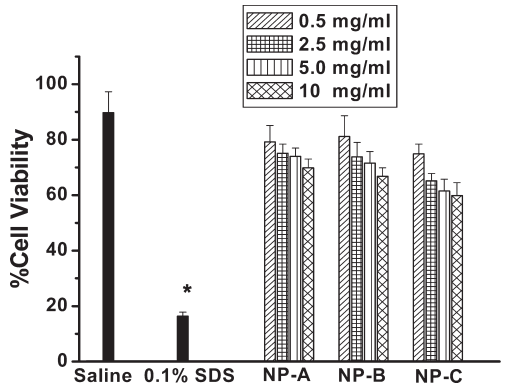

Figure 8. Effects of nanoparticulate PLA and PLGA formulations NP-A, NP-B and NP-C on the viability of Calu-3 cells. The test samples contained 0.5 mg/mL, 2.5 mg/mL, 5 mg/mL and 10 mg/mL of different formulations. Data represent mean ± SD, n = 8−16 (*results are significantly different from other treatment groups, $p < 0.05$).

NP-A, NP-B and NP-C, showed concentration-dependent increases in cytotoxicity. However, compared to the positive control 0.1% SDS all of the treatment groups showed high cell viability of between 65 and 80% for the different concentrations employed. Thus the data suggest that nanoparticulate formulations did not produce major damage to the pulmonary epithelium.

In summary, this is the first study that demonstrates that PLA and PLGA nanoparticles administered via the pulmonary route can be used for mucosal vaccination against hepatitis B. Humoral and mucosal immune responses produced by the formulations are functions of particle size and hydrophobicity of the polymers used. Uptake by dendritic cells may play a role in generating mucosal immunity upon administration of nanoparticles. However, a combination of factors including particle size, hydrophobicity and polymer type may play a role in generation of mucosal immune response.

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REFERENCES

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

(2) Diwan, M.; Tafaghodi, M.; Samuel, J. Enhancement of immune responses by co-delivery of a CpG oligodeoxynucleotide and tetanus toxoid in biodegradable nanospheres. *J. Controlled Release* **2002**, *85*, 247–62.

(3) Friede, M.; Aguado, M. T. Need for new vaccine formulations and potential of particulate antigen and DNA delivery systems. *Adv. Drug Delivery Rev.* **2005**, *57*, 325–31.

(4) Simonsen, L.; Kane, A.; Lloyd, J.; Zaffran, M.; Kane, M. Unsafe injections in the developing world and transmission of bloodborne pathogens: a review. *Bull. W.H.O.* **1999**, *77*, 789–800.

(5) Kane, A.; Lloyd, J.; Zaffran, M.; Simonsen, L.; Kane, M. Transmission of hepatitis B, hepatitis C and human immunodeficiency viruses through unsafe injections in the developing world: model-based regional estimates. *Bull. W.H.O.* **1999**, *77*, 801–7.

(6) Cui, Z.; Mumper, R. J. Intranasal administration of plasmid DNA-coated nanoparticles results in enhanced immune responses. *J. Pharm. Pharmacol.* **2002**, *54*, 1195–203.

(7) Mestecky, J. The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. Clin. Immunol.* **1987**, *7*, 265–76.

(8) Bivas-Benita, M.; van Meijgaarden, K. E.; Franken, K. L.; Junginger, H. E.; Borchard, G.; Ottenhoff, T. H.; Geluk, A. Pulmonary delivery of chitosan-DNA nanoparticles enhances the immunogenicity of a DNA vaccine encoding HLA-A*0201-restricted T-cell epitopes of Mycobacterium tuberculosis. *Vaccine* **2004**, *22*, 1609–15.

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

(10) Mundargi, R. C.; Babu, V. R.; Rangaswamy, V.; Patel, P.; Aminabhavi, T. M. Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. *J. Controlled Release* **2008**, *125*, 193–209.

(11) Thiele, L.; Merkle, H. P.; Walter, E. Phagocytosis of synthetic particulate vaccine delivery systems to program dendritic cells. *Expert Rev. Vaccines* **2002**, *1*, 215–26.

(12) Thiele, L.; Rothen-Rutishauser, B.; Jilek, S.; Wunderli-Allenspach, H.; Merkle, H. P.; Walter, E. Evaluation of particle uptake in human blood monocyte-derived cells in vitro. Does phagocytosis activity of dendritic cells measure up with macrophages?. *J. Controlled Release* **2001**, *76*, 59–71.

(13) Thomasin, C.; Corradin, C.; Men, Y.; Merkle, H. P.; Gander, B. Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: importance of polymer degradation and antigen release for immune response. *J. Controlled Release* **1996**, *41*, 131–145.

(14) Walter, E.; Dreher, D.; Kok, M.; Thiele, L.; Kiama, S. G.; Gehr, P.; Merkle, H. P. Hydrophilic poly(DL-lactide-co-glycolide) microspheres for the delivery of DNA to human-derived macrophages and dendritic cells. *J. Controlled Release* **2001**, *76*, 149–68.

(15) Jung, T.; Kamm, W.; Breitenbach, A.; Kaiserling, E.; Xiao, J. X.; Kissel, T. Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake?. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 147–60.

(16) Singh, J.; Pandit, S.; Bramwell, V. W.; Alpar, H. O. Diphtheria toxoid loaded poly-(epsilon-caprolactone) nanoparticles as mucosal vaccine delivery systems. *Methods* **2006**, *38*, 96–105.

(17) Foged, C.; Brodin, B.; Frokjaer, S.; Sundblad, A. Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. *Int. J. Pharm.* **2005**, *298*, 315–22.

(18) Xiang, S. D.; Scholzen, A.; Minigo, G.; David, C.; Apostolopoulos, V.; Mottram, P. L.; Plebanski, M. Pathogen recognition and development of particulate vaccines: does size matter?. *Methods* **2006**, *40*, 1–9.

(19) Shakweh, M.; Ponchel, G.; Fattal, E. Particle uptake by Peyer's patches: a pathway for drug and vaccine delivery. *Expert Opin. Drug Delivery* **2004**, *1*, 141–63.

(20) des Rieux, A.; Fievez, V.; Garinot, M.; Schneider, Y. J.; Preat, V. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. *J. Controlled Release* **2006**, *116*, 1–27.

(21) Katare, Y. K.; Muthukumaran, T.; Panda, A. K. Influence of particle size, antigen load, dose and additional adjuvant on the immune response from antigen loaded PLA microparticles. *Int. J. Pharm.* **2005**, *301*, 149–60.

(22) Newman, K. D.; Elamanchili, P.; Kwon, G. S.; Samuel, J. Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells in vivo. *J. Biomed. Mater. Res.* **2002**, *60*, 480–6.

(23) Borges, O.; Tavares, J.; de Sousa, A.; Borchard, G.; Junginger, H. E.; Cordeiro-da-Silva, A. Evaluation of the immune response following a short oral vaccination schedule with hepatitis B antigen encapsulated into alginate-coated chitosan nanoparticles. *Eur. J. Pharm. Sci.* **2007**, *32*, 278–90.

(24) Jaganathan, K. S.; Vyas, S. P. Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally. *Vaccine* **2006**, *24*, 4201–11.

(25) Makidon, P. E.; Bielinska, A. U.; Nigavekar, S. S.; Janczak, K. W.; Knowlton, J.; Scott, A. J.; Mank, N.; Cao, Z.; Rathinavelu, S.; Beer, M. R.; Wilkinson, J. E.; Blanco, L. P.; Landers, J. J.; Baker, J. R., Jr. Preclinical evaluation of a novel nanoemulsion-based hepatitis B mucosal vaccine. *PLoS ONE* **2008**, *3*, e2954.

(26) Thomas, C.; Gupta, V.; Ahsan, F. Particle size influences the immune response produced by hepatitis B vaccine formulated in inhalable particles. *Pharm. Res.* **2010**, *27*, 905–19.

(27) Thomas, C.; Gupta, V.; Ahsan, F. Influence of surface charge of PLGA particles of recombinant hepatitis B surface antigen in enhancing systemic and mucosal immune responses. *Int. J. Pharm.* **2009**, *379*, 41–50.

(28) Bharali, D. J.; Pradhan, V.; Elkin, G.; Qi, W.; Hutson, A.; Mousa, S. A.; Thanavala, Y. Novel nanoparticles for the delivery of recombinant hepatitis B vaccine. *Nanomedicine* **2008**, *4*, 311–7.

(29) Borges, O.; Cordeiro-da-Silva, A.; Romeijn, S. G.; Amidi, M.; de Sousa, A.; Borchard, G.; Junginger, H. E. Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination. *J. Controlled Release* **2006**, *114*, 348–58.

(30) Chong, C. S.; Cao, M.; Wong, W. W.; Fischer, K. P.; Addison, W. R.; Kwon, G. S.; Tyrrell, D. L.; Samuel, J. Enhancement of T helper type 1 immune responses against hepatitis B virus core antigen by PLGA nanoparticle vaccine delivery. *J. Controlled Release* **2005**, *102*, 85–99.

(31) Wendorf, J.; Singh, M.; Chesko, J.; Kazzaz, J.; Soewanan, E.; Ugozzoli, M.; O'Hagan, D. A practical approach to the use of nanoparticles for vaccine delivery. *J. Pharm. Sci.* **2006**, *95*, 2738–50.

(32) Florence, A. T. The oral absorption of micro- and nanoparticulates: neither exceptional nor unusual. *Pharm. Res.* **1997**, *14*, 259–66.

(33) Rawat, A.; Majumder, Q. H.; Ahsan, F. Inhalable large porous microspheres of low molecular weight heparin: in vitro and in vivo evaluation. *J. Controlled Release* **2008**, *128*, 224–32.

(34) Gupta, P. N.; Mahor, S.; Rawat, A.; Khatri, K.; Goyal, A.; Vyas, S. P. Lectin anchored stabilized biodegradable nanoparticles for oral immunization 1. Development and in vitro evaluation. *Int. J. Pharm.* **2006**, *318*, 163–73.

(35) Jaganathan, K. S.; Singh, P.; Prabakaran, D.; Mishra, V.; Vyas, S. P. Development of a single-dose stabilized poly(D,L-lactic-co-glycolic acid) microspheres-based vaccine against hepatitis B. *J. Pharm. Pharmacol.* **2004**, *56*, 1243–50.

(36) Yeh, M. K.; Coombes, A. G.; Chen, J. L.; Chiang, C. H. Japanese encephalitis virus vaccine formulations using PLA lamellar and PLG microparticles. *J. Microencapsul.* **2002**, *19*, 671–82.

(37) Zhao, A.; Rodgers, V. G. Using TEM to couple transient protein distribution and release for PLGA microparticles for potential use as vaccine delivery vehicles. *J. Controlled Release* **2006**, *113*, 15–22.

(38) Thomas, C.; Rawat, A.; Bai, S.; Ahsan, F. Feasibility study of inhaled hepatitis B vaccine formulated with tetradecylmaltoside. *J. Pharm. Sci.* **2008**, *97*, 1213–23.

(39) Debin, A.; Kravtzoff, R.; Santiago, J. V.; Cazales, L.; Sperandio, S.; Melber, K.; Janowicz, Z.; Betbeder, D.; Moynier, M. Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses. *Vaccine* **2002**, *20*, 2752–63.

(40) Vyas, S. P.; Singh, R. P.; Jain, S.; Mishra, V.; Mahor, S.; Singh, P.; Gupta, P. N.; Rawat, A.; Dubey, P. Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B. *Int. J. Pharm.* **2005**, *296*, 80–6.

(41) Evora, C.; Soriano, I.; Rogers, R. A.; Shakesheff, K. N.; Hanes, J.; Langer, R. Relating the phagocytosis of microparticles by alveolar macrophages to surface chemistry: the effect of 1,2-dipalmitoylphosphatidylcholine. *J. Controlled Release* **1998**, *51*, 143–52.

(42) Jalil, R.; Nixon, J. R. Microencapsulation using poly (L-lactic acid) III: Effect of polymer molecular weight on the microcapsule properties. *J Microencapsul.* **1990**, *7*, 41–52.

(43) Mittal, G.; Sahana, D. K.; Bhardwaj, V.; Ravi Kumar, M. N. Estradiol loaded PLGA nanoparticles for oral administration: effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo. *J. Controlled Release* **2007**, *119*, 77–85.

(44) Witschi, C.; Doelker, E. Influence of the microencapsulation method and peptide loading on poly(lactic acid) and poly(lactic-co-glycolic acid) degradation during in vitro testing. *J. Controlled Release* **1998**, *51*, 327–41.

(45) Li, M.; Rouaud, O.; Poncelet, D. Microencapsulation by solvent evaporation: state of the art for process engineering approaches. *Int. J. Pharm.* **2008**, *363*, 26–39.

(46) Garinot, M.; Fievez, V.; Pourcelle, V.; Stoffelbach, F.; des Rieux, A.; Plapied, L.; Theate, I.; Freichels, H.; Jerome, C.; Marchand-Brynaert, J.; Schneider, Y. J.; Preat, V. PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination. *J. Controlled Release* **2007**, *120*, 195–204.

(47) Nayak, B.; Panda, A. K.; Ray, P.; Ray, A. R. Formulation, characterization and evaluation of rotavirus encapsulated PLA and PLGA particles for oral vaccination. *J. Microencapsul.* **2009**, *26*, 154–65.

(48) Coombes, A. G.; Yeh, M. K.; Lavelle, E. C.; Davis, S. S. The control of protein release from poly(DL-lactide co-glycolide) microparticles by variation of the external aqueous phase surfactant in the water-in oil-in water method. *J. Controlled Release* **1998**, *52*, 311–20.

(49) Sahoo, S. K.; Panyam, J.; Prabha, S.; Labhasetwar, V. Residual polyvinyl alcohol associated with poly (D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. *J. Controlled Release* **2002**, *82*, 105–14.

(50) Takada, S.; Yamagata, Y.; Misaki, M.; Taira, K.; Kurokawa, T. Sustained release of human growth hormone from microcapsules prepared by a solvent evaporation technique. *J. Controlled Release* **2003**, *88*, 229–42.

(51) Gasper, M. M.; Blanco, D.; Cruz, M. E.; Alonso, M. J. Formulation of L-asparaginase-loaded poly(lactide-co-glycolide) nanoparticles: influence of polymer properties on enzyme loading, activity and in vitro release. *J. Controlled Release* **1998**, *52*, 53–62.

(52) Gilding, D. K.; Reed, A. M. Biodegradable polymers for use in surgery-polyglycolic/poly(lactic acid) homo-and copolymers:1. *Polymer* **1979**, *20*, 1459–64.

(53) Mehta, A. K.; Yadav, K. S.; Sawant, K. K. Nimodipine loaded PLGA nanoparticles: formulation optimization using factorial design, characterization and in vitro evaluation. *Curr. Drug Delivery* **2007**, *4*, 185–93.

(54) Lee, W. K.; Park, J. Y.; Yang, E. H.; Suh, H.; Kim, S. H.; Chung, D. S.; Choi, K.; Yang, C. W.; Park, J. S. Investigation of the factors influencing the release rates of cyclosporin A-loaded micro- and nanoparticles prepared by high-pressure homogenizer. *J. Controlled Release* **2002**, *84*, 115–23.

(55) Argentini, C.; Giuseppetti, R.; D'Ugo, E.; La Sorsa, V.; Tritarelli, E.; Orobello, S.; Canitano, A.; Gluck, R.; Rapicetta, M. A pre-S/S CHO-derived hepatitis B virus vaccine protects woodchucks from WHV productive infection. *Vaccine* **2005**, *23*, 3649–56.

(56) Espuelas, S.; Gamazo, C.; Balnco-Prieto, M. J.; Irache, J. M. Nanoparticles as Adjuvant-Vectors for Vaccination. In *Nanoparticulate Drug Delivery Systems*; Thassu, D., Deelers, M., Pathak, Y., Eds.; Informa Healthcare, USA, Inc.: New York, 2007; Vol. 166, p 317.

(57) Reece, J. C.; Vardaxis, N. J.; Marshall, J. A.; Crowe, S. M.; Cameron, P. U. Uptake of HIV and latex particles by fresh and cultured dendritic cells and monocytes. *Immunol. Cell Biol.* **2001**, *79*, 255–63.

(58) Yoshida, M.; Babensee, J. E. Molecular aspects of microparticle phagocytosis by dendritic cells. *J. Biomater. Sci., Polym. Ed.* **2006**, *17*, 893–907.

(59) Kanchan, V.; Panda, A. K. Interactions of antigen-loaded polylactide particles with macrophages and their correlation with the immune response. *Biomaterials* **2007**, *28*, 5344–57.