POTENTIAL CLINICAL RELEVANCE

Nanomedicine: Nanotechnology, Biology, and Medicine
9 (2013) 523–531

Original Article

nanomedjournal.com

Development of a nanoparticle-based influenza vaccine using the PRINT® technology

Ashley L. Galloway, PhD[a,1], Andrew Murphy, PhD[a,1], Joseph M. DeSimone, PhD[b,c,d], Jie Di, PhD[a], Jennifer P. Herrmann, BS[a], Michael E. Hunter, MS[a], Jeffrey P. Kindig, MS[a], Frank J. Malinoski, MD, PhD[a,*], Megan A. Rumley, BS[a], Daria M. Stoltz, BS[a], Thomas S. Templeman, PhD[a], Bolyn Hubby, PhD[a]

[a]*Liquidia Technologies, Inc., Research Triangle Park, NC, USA*
[b]*Department of Chemistry and Institute for Nanomedicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*
[c]*Department of Pharmacology and Institute for Nanomedicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*
[d]*Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC, USA*

Received 11 June 2012; accepted 2 November 2012

Abstract

Historically it is known that presentation of vaccine antigens in particulate form, for a wide range of pathogens, has clear advantages over the presentation of soluble antigen alone [J.C. Aguilar, E.G. Rodriguez, Vaccine adjuvants revisited. Vaccine 25 (2007) 3752–3762, M. Singh, D. O'Hagan, Advances in vaccine adjuvants. Nature Biotechnology 17 (1999) 1075–1081]. Herein we describe a novel particle-based approach, which independently controls size, shape, and composition to control the delivery and presentation of vaccine antigen to the immune system. Highly uniform particles were produced using a particle molding technology called PRINT® (Particle Replication in Non-wetting Templates) which is an off-shoot of imprint lithography [J Am Chem Soc 127 (2005) 10096–10100, J Am Chem Soc 126 (2004) 2322–2323, Chem Soc Rev 35 (2006) 1095–1104, J Am Chem Soc 130 (2008) 5008–5009, J Am Chem Soc 130 (2008) 5438–5439, Polymer Reviews 47 (2007) 321–327, Acc Chem Res 41 (2008) 1685–1695, Acc Chem Res 44 (10) (2011) 990–998]. Cylindrical (diameter [d]=80 nm, height [h]=320 nm) poly (lactide-co-glycolide) (PLGA) based PRINT particles were designed to electrostatically bind commercial trivalent injectable influenza vaccine. In a variety of blended PLGA formulations, these particles were safe and showed enhanced responses to influenza hemagglutinin in murine models.

From the Clinical Editor: Shape is one of the determining factors in interactions of nanoparticles with their biologic environment. PRINT technology is able to fabricate nearly uniform nanoparticles and this technology is tested here in murine models to effectively deliver influenza vaccine.

Key words: Nanotechnology; Influenza vaccine; PRINT; Soft lithography; Hemagglutinin

Several tools exist to generate nanoparticle systems[1–3] including chemical conjugation, liposomes, lipid nanoparticles,[4] emulsion systems,[5] viruses, dendrimers, and micelles. These systems have greatly enhanced the fields of vaccines and drug delivery, but several hurdles remain.[6] A major technical barrier with these technologies is that they do not have the flexibility to independently and simultaneously control particle size, shape, and composition. Thus, while one technique may be applicable to one antigen or drug, the breadth of use is often limited. For instance, changes in composition can significantly impact the size, shape, dynamics, and even functionality of the resulting nanoparticle, particularly in self-assembled systems.[7,8] However, precise control over the composition, size, shape, and surface chemistry of micro and nanoparticles is possible with the PRINT® technology. Using the PRINT technology, particles can be generated through a broad range of target sizes (e.g., 20 nm to >100 microns), shapes (e.g., rods, filamentous, discs, toroids), compositions (e.g., organic/inorganic, solid/porous),

Conflict of Interest Statement: The authors on this manuscript are current or former employees of Liquidia Technologies, Inc. Joseph M. DeSimone is the founder of Liquidia Technologies, Inc.

All sources of support for Research: This work was funded by Liquidia Technologies, Inc.

*Corresponding author: P.O. Box 110085 Research Triangle Park, NC 27709, USA.
E-mail address: Frank.Malinoski@Liquidia.com (F.J. Malinoski).

[1] Contributed equally to this work.

1549-9634/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.nano.2012.11.001

cargo (e.g. oligonucleotides, small molecule drugs, proteins, or imaging agents) and surface properties (e.g., protein complexes, targeting antibodies, cationic/anionic charges).[7,9–15] The ability to independently control these parameters in the development of nanomedicines may bring the potential to develop particle-based therapeutics in areas not previously possible.

Vaccines are an attractive field for nanotechnology as it is historically known that the presentation of antigens in particulate form, for a wide range of pathogens, has clear advantages over the presentation of soluble antigen alone.[16,17] This is evidenced by the success of vaccines such as the hepatitis B vaccine, the human papilloma virus vaccine, the inactivated polio vaccine and others. And while the influenza hemagglutinin surface glycoprotein forms rosettes under certain conditions, the immunogenicity and protection afforded by HA vaccines has some limitations.[18] Thus it is hypothesized that the delivery of antigen(s) adsorbed to a bioabsorbable particle will increase humoral responses through more efficient crosslinking of receptors on B cells,[1] and will also increase cellular responses by mediating more efficient uptake by antigen-presenting cells. Antigen adsorption to nanoparticles may also decrease the amount of antigen necessary to induce an immune response, and thereby provide a significant dose sparing. Unlike traditional live, attenuated vaccines, the PRINT technology has the ability to utilize the nanoscale size of viral vaccines combined with a potentially safer and more controllable synthetic approach, avoiding many of the safety concerns of traditional live virus vaccine delivery.

While the immunogenicity and efficacy of influenza HA in the prevention of seasonal influenza is well established, gaps exist in the protection of the elderly and questions remain regarding the optimal approach and formulations for protection against pandemic strains.[19,20] Thus, the influenza HA was chosen as a target to investigate the impact of PRINT particles on the immune response relative to soluble antigen.

The work described herein explores the combination of PRINT particles that bind influenza antigens to understand if delivery of the vaccine antigen adsorbed to a simple bioabsorbable particle is safe and will increase vaccine effectiveness and/or reduce the amount of antigen necessary to induce an immune response. Such data, with particles manufactured under current Good Manufacturing Practice could then be considered for clinical evaluation to determine the extent to which this technology might address the unmet needs in the prevention of influenza.

Methods

Materials

Poly(lactic-co-glycolic acid) (PLGA) (MW 30,000 Daltons) was purchased as USP grade material (5050DLG3A) from Lakeshore Biomaterials (Birmingham, AL, USA Cat# LP345). Poly(2-dimethylamino)ethyl methacrylate (pDMAEMA) (MW 50,000 Daltons) was purchased as research grade material from Polysciences, Inc. (Warrington, PA USA Cat# 21510). Cholesteryl N-(trimethylammonioethyl)carbamate chloride (TC-cholesterol), approximately 95% pure was purchased from Sigma-Aldrich Chemical Company (St Louis, MO USA Cat# C9446). 3β-[N-(N′,N′-Dimethylaminoethane)-carbamoyl] Cholesterol Hydrochloride (DC-cholesterol) was obtained from Avanti Polar Lipids, Inc (Alabaster, AL USA Cat# 770001) as GMP grade material. D-Mannitol ((2R,3R,4R,5R)-hexane-1,2,3,4,5,6-hexol) USP grade was purchased from Spectrum Chemicals (Gardena, CA, USA Cat# MA-165). Polyvinyl alcohol, USP grade (PVOH) was also purchased from Spectrum Chemicals (Gardena, CA, USA Cat# P1180). Plasdone S-630®, USP grade was obtained from International Specialty Products (Wayne, NJ, USA Cat# 72473I). Seasonal trivalent influenza vaccines (TIVs) were purchased from commercial distributors: 2009–2010 and 2010–2011 Fluzone® (Sanofi-Pasteur), 2008–2009 and 2010–2011 Fluvirin® (Novartis), 2009–2010 AgriFlu® (Novartis), 2009–2010 Afluria® (Merck). Recombinant HA's were purchased from either Protein Sciences (Meriden, CT, USA Cat# 3006) or Immune Tech (Foster City, CA, USA): HA(ΔTM)(B/Brisbane/60/2008) (Cat# IT-003-B3p), HA(ΔTM)(H1N1)(A/Brisbane/59/2007) (Cat# IT-003-0012ΔTMp), HA(ΔTM)(H3N2)(A/Brisbane/10/2007) (Cat# IT-003-0042ΔTMp), HA(ΔTM)(H1N1)(A/California/06/2009) (Cat# IT-003-SW1ΔTMp), HA(ΔTM)(B/Florida/04/2006) (Cat# IT-003-B2p). Phosphate buffered saline (PBS) was purchased from Invitrogen (Cat# 20012–043). Hemagglutinin (HA) specific antibodies used for western blots were purchased from Protein Sciences (Meriden, CT): Polyclonal anti-Rabbit H3 (Cat# 6100), H1 (Cat# 6000), or B (Cat# 6200). Anti-Rabbit IgG for western blots and ELISAs were purchased from Jackson Immuno-Research (Cat# 111-055-046), Anti-Mouse IgG for ELISAs was purchased from Sigma (Cat# A3688), NBT/BCIP substrate solution for western blots was purchased from Thermo Fisher Scientific (Cat# PI-34042) and SIGMAFAST tablets (p-Nitrophenyl phosphate) developer for ELISAs was purchased from Sigma (Cat# N2770-50SET).

Preparation of PRINT particles

Solutions of PLGA and cationic additives were generated by mixing a solution of PLGA in DMF with solutions of pDMAEMA, TC-Cholesterol, or DC-Cholesterol in either methanol or chloroform. With these solutions, cylindrical (diameter [d] = 80 nm, height [h] = 320 nm) cationic PLGA-based nanoparticles were prepared using the PRINT technology as described previously.[7,14,21,22] Particles were harvested onto a PET film coated with Plasdone S-630®. The harvested particles were passed through an acidic 0.01 wt% PVOH solution in water to dissolve the plasdone and release particles into suspension. The particle suspension was sterilized using a 0.2 μm polyether sulfone membrane (Millipore, Billerica, MA USA) and purified by tangential flow filtration with a 0.05 μm polysulfone hollow fiber membrane (Spectrum Laboratories, Inc. Rancho Dominguez, CA USA). For larger batches, particles were formulated with mannitol, filled into two milliliter vials (Wheaton Science Products, Millville, NJ USA) and lyophilized using an FTS Systems Lyostar I Tray Lyophilizer (SP Scientific, Warminster, PA, USA).

PRINT particle characterization methods

PRINT particles in suspensions were diluted in water to a range between 0.01 to 0.1 mg/mL for size and/or zeta potential

analysis. Particle size and zeta potential measurements were determined using a Zetasizer Nano (Malvern, Worchestershire, UK). Particle concentration was determined gravimetrically. Scanning electron microscopy images were recorded on a Hitachi S-4700 Cold Cathode Field Emission Scanning Electron Microscope. Protein binding capacity was determined by first mixing particles and influenza hemagglutinin (HA) antigens and incubating the suspension at 4 °C for 20 minutes. The supernatant was then separated from the particles and measured for remaining protein by the standard Bradford assay methodology using a plate reader with quantification via a HA protein standard curve generated from the HA antigen source used in binding experiments. In addition to total binding, a western blot was used to confirm the binding of each of the three HA antigens contained in the trivalent influenza vaccine (TIV), Fluzone®, to the particles. As with the Bradford assay, particles (10 to 40 μg) were mixed with 1 μg Fluzone and incubated at 4 °C for 20 minutes. Particles were then separated from the suspension, washed with PBS, and exposed to surfactant solutions to detach bound protein from the particles. These samples were loaded onto 4–20% Tris-glycine gels, electrophoresed, and blotted onto PVDF membranes. Western blots were performed using HA specific primary antibodies and visualized using an NBT/BCIP substrate.

Evaluation of immune responses

All animal studies were conducted in compliance with the Animal Welfare Act Regulations, 9 CFR 1–4, and animals were handled and treated according to the Guide for the Care and Use of Laboratory Animals.[23] For immunogenicity evaluations each mouse study group consisted of typically 6 BALB/c female mice at 6 to 8 weeks old expected to be influenza naive. Each of the rabbit study groups contained 18 New Zealand White (NZW) rabbits, both male and female, at 6.5 months of age. Animals were immunized intramuscularly (IM) in the hind leg with nanoparticle vaccines suspended in 5% mannitol / 0.1 wt% PVOH solution at days 1 and 22. Animals were bled prior to the prime and boost injections (day 0, day 21) and at 1 week and 2 weeks post-boost (day 29, day 36). Sera were then assessed for HA-specific antibodies by both enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HAI). The ELISA method was based on general protocols[24] using MaxiSorp™ plates coated with strain-specific, recombinant HA as the capture reagent. Detection was performed using p-nitrophenyl phosphate as the substrate for alkaline-phosphatase conjugated goat anti-mouse IgG antibody (whole molecule for mouse studies) or goat anti-rabbit IgG antibody (Fc-gamma-specific for rabbit studies). A visible adsorption at 405 nm was recorded using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). Reciprocal titers for each sample were defined by the serum dilution that had an optical density of 0.2, where background was reading 0.0. The HAI assay was developed using the protocol from the WHO Manual on Animal Influenza.[25] Briefly, dilutions of animal sera were incubated with a constant concentration of either live or attenuated influenza virus (strain-specific) and Turkey red blood cells (CBT Farms or Lampire Biological Products). Plates were then tilted at a 45° angle and inhibition of agglutination was observed, where the HAI titer was determined as the dilution factor of the last well with complete inhibition. Data from immunoassays was analyzed with either Microsoft® Excel 2007 (Microsoft Corporation, Redmond, WA, USA) or GraphPad PRISM 5 (GraphPad Software, Inc, La Jolla CA, USA). All immunological data is presented as the geometric mean of the reciprocal titer with error bars indicating the standard error of the mean. Statistical analyses of data were completed using JMP 8 (SAS Cary, NC, USA) and statistical significance between data was determined using a Student's t-test[26] ($P<0.05$).

Toxicology evaluation

Animals included in the murine immunogenicity studies described above were also evaluated for toxicological effects through body weight measurements, clinical observations, mortality, and in some studies histopathological and/or cytokine analysis. Each animal was observed and weighed before Day 0 for randomization, prior to initial vaccine/dose formulation administration, weekly thereafter, and prior to humane euthanasia. Blood was collected via the retro-orbital sinus for survival blood collection. Animals were humanely euthanized, and blood was collected via the vena cava two to three weeks following the second dose administration. For histopathologic evaluation, the bilateral hind leg muscle, including the sciatic nerve, was collected at necropsy and preserved in 10% neutral buffered formalin (NBF). The left hind leg was histologically processed and embedded, sectioned, stained with hematoxylin and eosin, and microscopically examined. The right hind leg muscle was held for possible histopathological evaluation. Data was analyzed using Microsoft Excel 2007, GraphPad PRISM 5.0, or for statistical analyses, JMP 8 (SAS Cary, NC, USA). Statistical significance between data was determined using a one-way ANOVA followed by a Tukey post test.

A GLP toxicology study was conducted in 6.5-month old male and female NZW rabbits (MPI Research). Rabbits (18 per group; 9 per sex in each group) were immunized IM on Days 1 and 22 with PBS, a standard adult dose of Fluzone, Fluzone + 1.8 mg LIQ001, Fluzone + 5.4 mg LIQ001, or 5.4 mg LIQ001 alone. Animals were evaluated for the reversibility, progression, or delayed appearance of any observed toxicological changes 24 hours following the boost dose (treatment phase) and following a 3-week post-boost observation period (recovery phase). Body weights, temperature, food consumption, hematology, coagulation, clinical chemistry, C-reactive protein (CRP), serum protein electrophoresis (SPE), and organ weights were analyzed via group pair-wise comparisons using either Levene's test,[27] Dunnett's comparison,[28] or Welch's t-test.[26] Leukocyte counts were log transformed and then analyzed by the group pair-wise comparisons described above. Urinalysis was first rank transformed and then analyzed with Dunnett's test or a Student's t-test.[26] Formation of erythema, eschar, or edema was analyzed by Cochran Mantel Haenszel[29] test. Results of all pair wise comparisons were reported at the 0.05 and 0.01 significance levels. All endpoints were analyzed using two-tailed tests.

Results

PLGA, poly(lactic-co-glycolic acid), was selected as the major component of the particles because it has broad use in human products[30] to include: drug delivery,[31] sutures,[32] implants, prosthetic devices, and scaffolds for tissue engineering.[32,33] Particle size and shape were chosen to be cylindrical particles with d= 80 nm and h=320 nm due in part to the ability to apply sterile filtration to this PRINT particle shape.

The general vaccine design was to establish a PLGA based particle without added adjuvant to which TIV would be added to previously fabricated particles at or near the time of animal injection and the immunologic components (primarily HA) would electrostatically bind to the particles. In order to achieve binding of TIV components to the particles, cationic small molecules or polymers were added in various amounts (≤10% by weight) to a solution of PLGA that would be used to fabricate particles. Particles as shown in Figure 1, *A*, were manufactured alone and then mixed with soluble HA antigens from the seasonal influenza vaccine prior to use. Not surprisingly, as the amount of cationic additive increases, the zeta potential of the blended PLGA based PRINT particle increases (data in supplemental information). After generating multiple compositions, it was found that the degree of HA antigen associated with the blended PLGA particle correlated with the charge of the particle (Figure 1, *B*). Particles with a positive surface charge (zeta potential >0 mV) showed substantially greater binding of the vaccine HA antigens than those with a negative surface charge (zeta potential <0 mV). The use of three different cationic additives, pDMAEMA, TC-cholesterol, or DC-cholesterol blended separately into cylindrical 80×320 nm PLGA-based particles, resulted in particles with comparable positive zeta potentials (+20 mV). Similar HA binding profiles were observed when a constant antigen dose was exposed to increasing amounts of these three particles at the same zeta potential (Figure 1, *C*). Moreover, similar plateaus on each binding curve were reached after all available protein was bound to the particles.

In addition to measuring total protein binding, western blot was used to confirm that each of the three HA antigens contained in the trivalent influenza vaccine were bound to the particles. Two different doses of particles (10 and 40 μg) were evaluated with 1 μg of Fluzone. The blot in Figure 2 shows that more of Fluzone B strain antigens were bound with the higher dose of particles (40 μg), very little antigen was observed in the wash, and that HA antigen was easily detached from the particles with surfactant solutions, with or without reducing buffer. Similar results were seen with the H3N2 and H1N1 western blots (data not shown), indicating that antigens from all three strains were bound comparably.

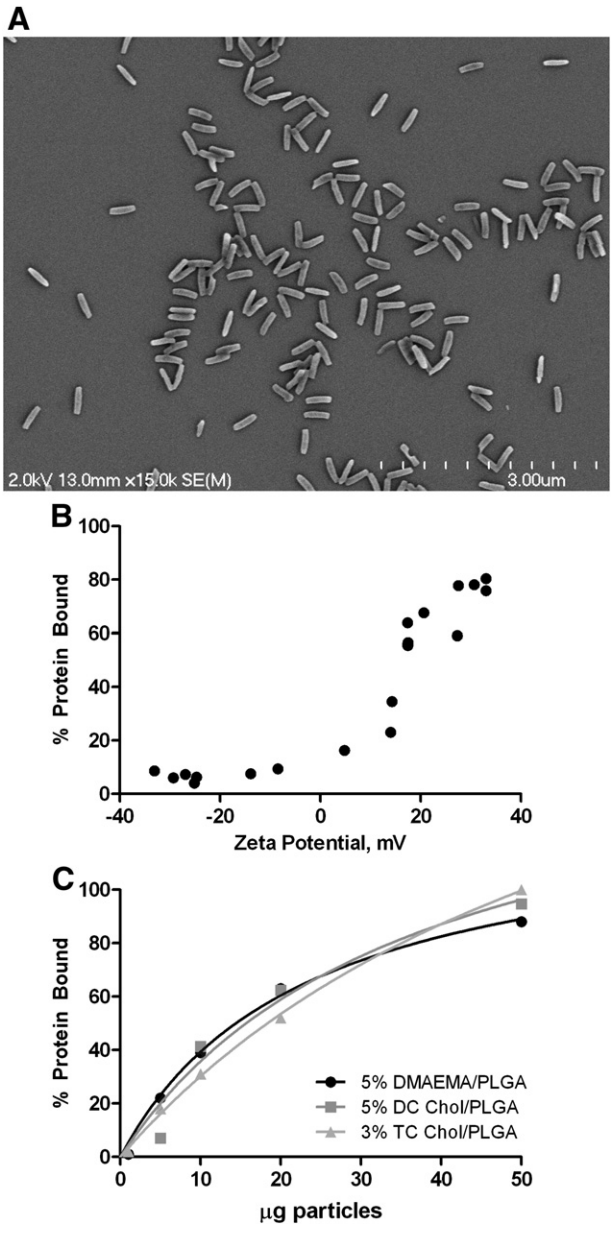

Figure 1. Protein adsorption to cationic cylindrical (d=80 nm; h=320 nm) PLGA particles. **(A**, left**)**: A scanning electron microscopy image of a cationic PLGA particle prior to the addition of HA. **(B**, middle**)**: Graph showing the correlation of the binding of 1 μg Fluvirin proteins to 20 μg of particles of various composition and surface charge (zeta potential) **(C**, right**)**. Binding (measured by Bradford assay) of 1 μg Fluvirin® antigen to increasing amounts of three PGLA-based particles with comparable particle surface charge (zeta potential, +20 mV) but containing different cationic agents.

Animal studies

A series of murine immunogenicity studies were performed to determine whether intramuscular injection of the PRINT-PLGA/TIV particles would enhance immune responses to HA as compared to soluble TIV. Initial studies evaluated the role of particle surface charge and type of additive using 1 μg of HA protein from a commercial TIV, Fluvirin (2008–09), adsorbed to three different PRINT particle compositions (Figure 3). Particle dose was chosen to be the amount needed to bind >70% of the Fluvirin dose. These compositions were injected as a mixture of particle-adsorbed and free antigen into BALB/c mice (prime at day 1, boost at day 22). ELISA results evaluating total IgG for all strains combined, at 1 week post-boost (day 29) demonstrated that

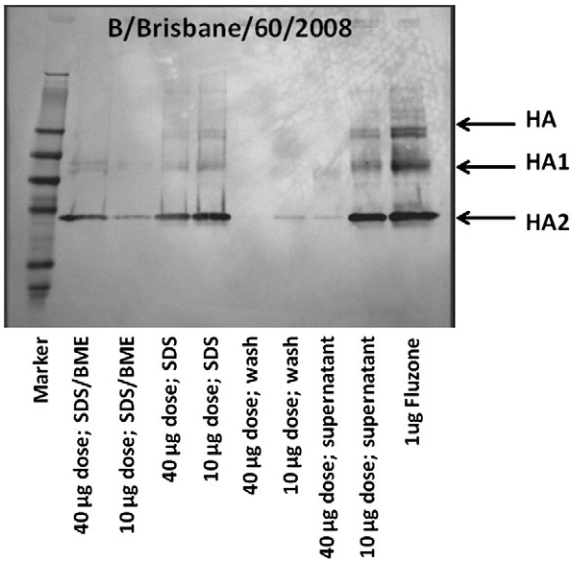

Figure 2. Western blot showing that B/Brisbane/60/2008 was adsorbed to cylindrical (d=80 nm; h=320 nm) cationic PLGA nanoparticles. 1 µg of Fluzone was exposed to either 10 µg (low dose) or 40 µg (high dose) of particles respectively. Particles were then exposed to sodium dodecyl sulfate (SDS) or SDS with β-mercaptoethanol (BME) to remove bound protein.

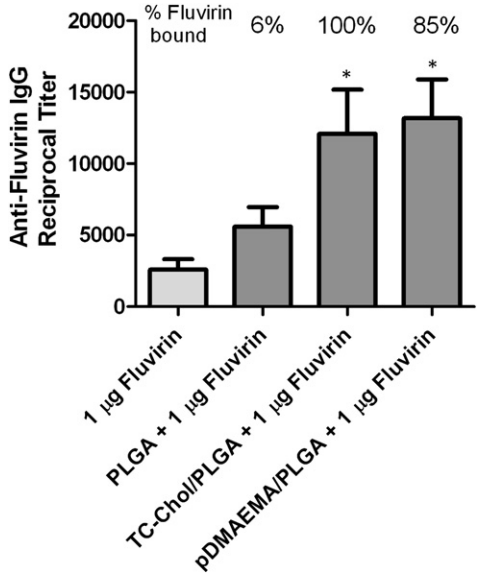

Figure 3. Effect of particle cationic additive on anti-HA IgG titers. Anti-HA (Fluvirin) IgG titers (GMT±SEM) in BALB/c mice after two injections with PLGA particles containing different cationic additives and adsorbed with Fluvirin. Data collected 1 week after boost (day 28). Statistical significance between each group and the TIV alone was determined using a Student's t-test[26] ($p<0.05$) and is indicated with an asterisk (*).

PRINT particles containing only PLGA (zeta potential, −40 mV) with 6% HA protein binding, yielded a 2-fold enhancement of anti-HA IgG titers compared with soluble Fluvirin. In contrast, PRINT particles containing the cationic additives TC-cholesterol (zeta potential, +32 mV) or pDMAEMA (zeta potential, +24 mV), each of which bound greater than 85% of the HA protein, displayed a 5-fold enhancement of anti-Fluvirin IgG titers compared with the soluble Fluvirin (Figure 3).

Subsequent murine immunogenicity studies tested responses to each of the three strains contained in TIV as compared to the soluble TIV control with 20 µg of particles comprised of 5% pDMAEMA in PLGA. As shown in Figure 4, the positively charged PLGA particles with adsorbed TIV showed significantly higher responses across all strains, including the influenza B strain.

In addition to evaluating the 2008–2009 Fluvirin TIV, cationic PLGA particles were mixed with other trivalent influenza vaccines including Fluzone, Agriflu®, and Afluria® (2009–2010 season); as well as Fluzone and Fluvirin (2010–2011 season). The particles showed comparable protein binding ability when mixed with any of the trivalent influenza vaccines and a similar improvement in ELISA antibody response as compared to the soluble TIV (data not shown). Additionally, previous strain specific responses (as generated in Figure 4) were further explored with the HAI assay in the current study. Functional antibody responses were measured by HAI and, as shown in Figure 5, there was a 4-fold improvement in HAI titers to the B strain of mice immunized with particle-TIV combination compared to mice immunized with TIV alone.

To assess the question of whether these increased responses would also be observed with a pandemic strain of influenza and therefore further establish the interchangeable nature of the base PRINT particle system, cationic PLGA-based PRINT particles were mixed with insect cell derived recombinant A/California/04/2009 (H1N1) HA protein. Six mice per group were immunized (prime day 1, boost day 22) with either recombinant H1N1 alone or in combination with cationic PLGA-based PRINT particles. As shown in Figure 6, *A*, at two weeks post-boost, a significantly higher ELISA antibody response was observed from the PRINT particle vaccine as compared to the soluble H1N1 protein.

To evaluate whether a lower dose of antigen in combination with PRINT particles could evoke an equivalent immune response to a higher dose of soluble antigen alone (dose sparing), cationic PLGA-based PRINT particles were combined with 1, 0.4, 0.1, or 0.025 µg of Fluvirin and injected intramuscularly into mice. At day 21, a stronger HA ELISA antibody response was seen from animals receiving PRINT particles+0.025 µg of Fluvirin than those receiving 1 µg of Fluvirin alone, representing a 40-fold decrease in antigen dose (Figure 6, *B*).

Toxicology studies

In addition to measuring the immunogenicity of PRINT particle antigen formulations, preclinical mouse studies also evaluated safety. In these previously described studies, a broad range of cationic PLGA PRINT particle masses (20 µg to 500 µg) were evaluated in mice with and without influenza HA antigen. No test-article-related effects regarding clinical observations, body weight changes, or mortality were noted. In one study, histopathology of the injection site was performed, showing small amounts of mostly acute mild inflammation surrounding the muscle and minimal amounts of chronic inflammation within the skeletal muscle (data in

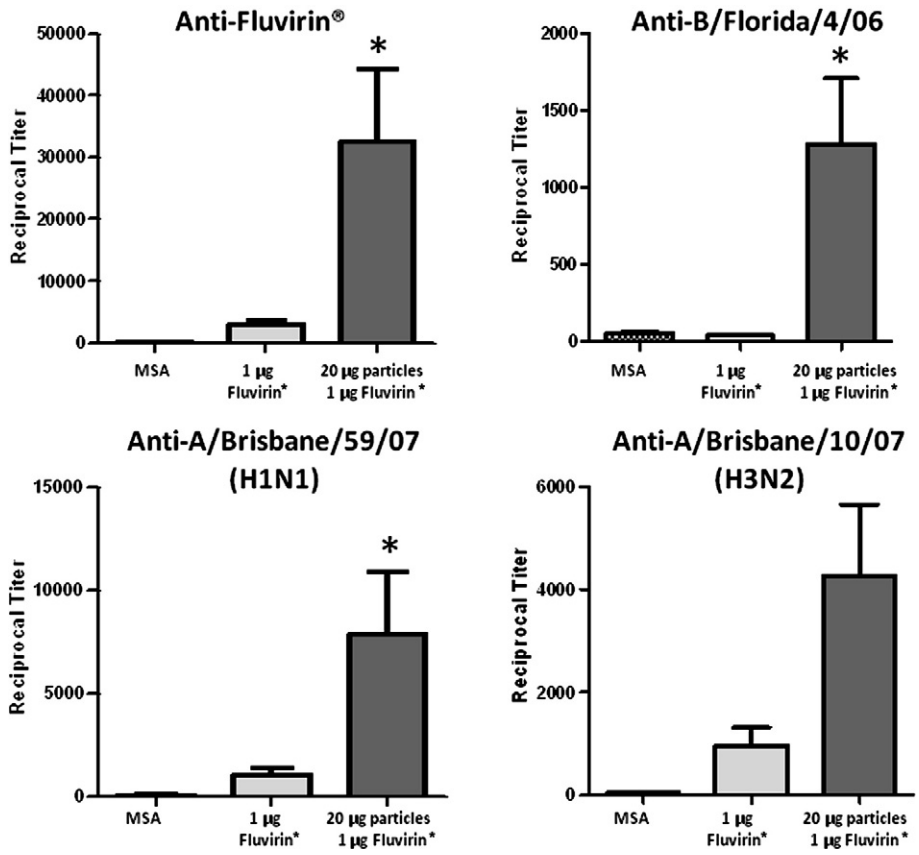

Figure 4. Cylindrical (d=80 nm; h=320 nm) cationic PLGA particles enhance strain-specific IgG titers. Each panel shows an IgG titer (GMT±SEM) (anti-Fluvirin or strain-specific) from BALB/c mice at 1 week post-boost (day 28) after two injections with 20 µg 5% DMAEMA/PLGA particles adsorbed with mouse serum albumin: MSA (control particles), 1 µg Fluvirin alone, or 1 µg Fluvirin adsorbed to 20 µg of 5% DMAEMA/PLGA particles. Statistical significance between each group and the TIV alone was determined using a Student's t-test[26] ($p<0.05$) and is indicated with an asterisk (*).

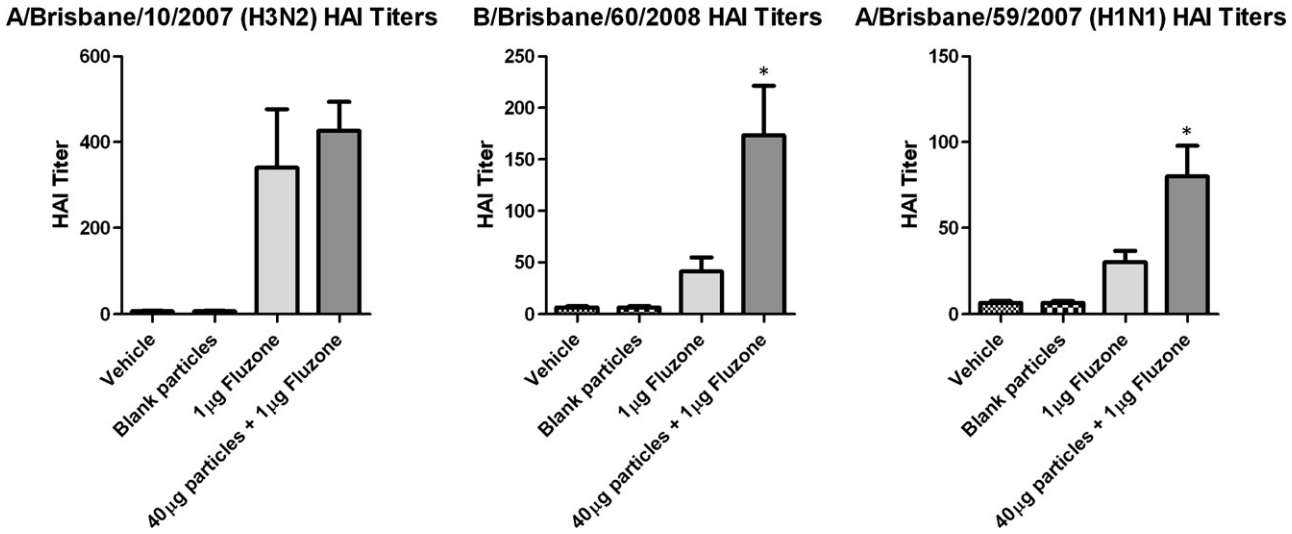

Figure 5. Functional antibody response measured by HAI. Each panel shows HAI titers 2 weeks post-boost after two injections with 40 µg of cylindrical (d=80 nm; h=320 nm) 5% DC Cholesterol / PLGA PRINT particles mixed with Fluzone prior to injection. Left to Right: H3N2, B, H1N1 HAI titers. Statistical significance between each group and the TIV alone was determined using a Student's t-test[26] ($p<0.05$) and is indicated with an asterisk (*).

supplemental material). In the same study, serum concentrations of seven pro-inflammatory cytokines were evaluated before immunization and at 24 hours after immunization (both prime and boost injections). Five cytokines (IFN-γ, IL-10, IL-6, CXCL-1/mKC, TNF-α) showed slight increases from pre-immunization values following prime and boost injections

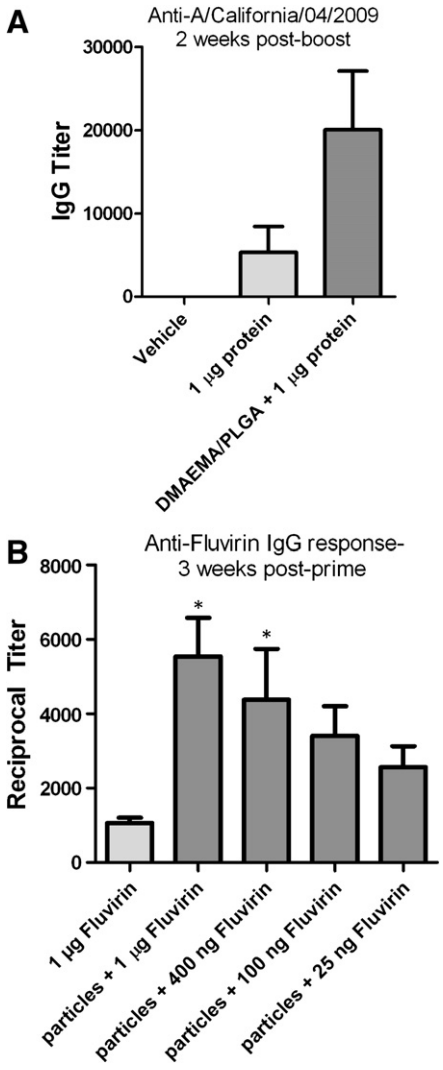

Figure 6. **(A)** Enhancement of antibody response to pandemic H1N1. Panel on the left shows IgG ELISA data from serum collected 2 weeks post-boost (day 35) after two injections with cylindrical (d=80 nm; h=320 nm) 5% DMAEMA/PLGA PRINT particles mixed with recombinant protein A/California/04/2009 prior to injection. **(B)** Dose sparing effect observed with Fluvirin in combination with cylindrical (d=80 nm; h=320 nm) cationic PRINT particles. Panel on the right shows IgG ELISA data from serum collected 3 weeks post prime (day 21) after one injection with 5% DMAEMA/PLGA PRINT particles of the same size mixed with Fluvirin prior to injection. Statistical significance between each group and the TIV alone was determined using a Student's t-test[26] ($p<0.05$) and is indicated with an asterisk (*).

(data in supplemental material). These increases were not found to be statistically significant (one-way ANOVA followed by a Tukey post test) and no consistent differences in cytokine levels between particle-treated groups and groups receiving Fluzone alone were observed. For toxicology studies, DC-cholesterol was selected as the cationic additive as it was safely used as a component of a liposomal formulation tested in a human gene therapy trial.[34–36] The particles for this development were labeled LIQ001. The final design of LIQ001 was determined to be a cylindrical particle (d=80 nm; h=320 nm) containing approximately 10% DC-cholesterol in PLGA intended to be combined with Fluzone within a few hours of immunization. A batch size for LIQ001 of 1–2 grams was chosen in order to meet preclinical, analytic, and clinical testing. Multiple batches were produced within a few weeks as needed for product development.

A GLP-compliant toxicology study was performed in 6.5-month old male and female NZW rabbits (9 per sex per group). The rabbits were immunized twice with one of the following: placebo (PBS), the standard human dose of Fluzone alone (45 μg HA), 45 μg of Fluzone adsorbed to 1.8 mg or 5.4 mg of LIQ001, or 5.4 mg LIQ001 alone. Animals were evaluated for the reversibility, progression, or delayed appearance of any observed toxicological changes 24 hours following the boost dose (treatment phase) and following a 3-week post-boost observation period (recovery phase). No test-article-related effects were noted regarding mortality, clinical observations, body weight, food consumption, body temperature, dermal irritation, urinalysis, organ weights, and macroscopic evaluations. Overall LIQ001 was well tolerated with evidence of dose-related transient injection site inflammation associated with LIQ001 combined with 45 μg Fluzone (data in supplemental information). Changes in spleen weight were observed in groups receiving Fluzone with or without LIQ001; however, no spleen weight change was observed in groups receiving LIQ001 alone. Based upon findings limited to inflammation, the No Observed Adverse Effect Level (NOAEL) of this study was 5.4 mg particles+45 μg Fluzone.

Discussion

These novel monodisperse nanoparticles were designed with the following considerations in mind: i) the presentation of vaccine antigens as particles is generally superior to soluble antigen[16,17]; ii) nanoparticles made of materials with known previous human use and human safety are preferred in new products; iii), such particles would bind the antigen of choice (influenza hemagglutinin antigen) and preserve, and potentially enhance, the immunogenicity of the antigen. Proof-of-concept studies demonstrated that the mixing of HA proteins from the trivalent influenza vaccine was optimal with cationic charged PRINT particles. Further, the binding of TIV components to these particles correlated with increased immune responses in naïve mice. The improvements were generally seen across all three influenza strains in the TIV preparations regardless of manufacturer or year of formulation. Importantly, these increases included improved responses to the B strain which has historically proven to be problematic in human trials of TIV. In addition, improvements and dose sparing were seen using recombinant H1N1 HA.

Because of the unique PRINT process, it was possible to independently evaluate the particle composition and surface charge, to explore the strain specificity of the immune response, to test the breadth of the antigen source, to probe the functionality of the antibodies produced, and to understand the dose sparing potential of this particulate vaccine.

Other PLGA-based particles have shown the ability to bind only 0.3 μg of HA antigen per mg of PLGA particles or 0.03% w/w.[18] However, by controlling the size, shape, and composition, PLGA-based PRINT particles were engineered to bind a very high dose of antigen to the particle surface. In fact, the lowest dose of particles advanced for testing, 0.45 mg, showed the ability to bind 60% of the adult seasonal influenza vaccine antigen dose, translating to a binding capacity of 60 μg of antigen per milligram of particles, or six weight percent antigen. This represents a 200-fold improvement in antigen binding for LIQ001 as compared to other PLGA based particles[18] which could translate into a lesser amount of product administered to deliver the intended antigen dose.

Until recently, alum was the only adjuvant that was used in a licensed vaccine in the USA[37] and historically aluminum salts have not been able to enhance responses to influenza vaccines.[38] In late 2009, the AS04 containing vaccine, Cervarix™ was licensed for use in the USA.[39] This adjuvant, along with others such as AS03 and MF59 have been in approved vaccines in Europe for several years now, with the approval of Fluad® containing MF59 in 1997.[40] The benign nature of LIQ001 particles in preclinical toxicology studies suggests that these particles may improve antigen presentation but may not induce cytokine responses seen with other adjuvants. However, the versatility of the PRINT technology is expected to allow for the incorporation of adjuvants as additional cargo within the particle and/or on the surface of the particle to further enhance immune responses. The freedom in product design offered could give rise to PRINT particle vaccine products with multiple adjuvants in and/or on separate particles or combined with antigen. Through the use of multiple targeting mechanisms such as size, shape, and surface ligands, potentially harmful systemic exposure of adjuvant containing particles could be limited, allowing for a focused delivery to the desired location.

The PRINT technology offers new capabilities to generate highly controlled particle-based vaccines. Multiple batches were prepared during preclinical studies which yielded monodisperse particles with comparable chemical contents, physical characteristics, and protein binding ability. LIQ001, the first generation of PRINT particle vaccines, has enabled Liquidia to demonstrate GMP manufacturing capabilities for the PRINT technology. Unlike other technologies, the flexibility and breadth of the PRINT technology provides a platform to explore the inclusion of additional immunostimulatory molecules, the novel ability to test the impact of size and shape of particles on the immune response in future vaccine products, and furthermore, has the potential to develop particle based therapeutics in areas not previously possible.

Acknowledgments

The authors would like to acknowledge several contributors to the data included in this paper. We appreciate the help of Joel A. Cohen, Virginia S. Conley, Christopher S. Hinson, Joseph R. Marchand, Mark A. Patrick, RiLee H. Robeson, Samantha B. Roth, and Robin Spivey for their work in physical and biological characterization of the product. Nanoparticle manufacturing support was greatly appreciated from M. Shawn Hunter, Sean Meng, Casey Shanley, and Jesse T. White. The authors would also like to thank David E. Jensen for regulatory support, Jennie H. Orr for strategic program guidance, and Jonathan F. Smith for scientific oversight.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2012.11.001.

References

1. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* 2010;**10**:787-96.
2. Shahiwala A, Vyas TK, Amiji MM. Nanocarriers for systemic and mucosal vaccine delivery. *Recent Pat Drug Deliv Formul* 2007;**1**:1-9.
3. Saroja CH, Lakshmi PK, Bhaskaran S. Recent trends in vaccine delivery systems: a review. *Int J Pharma Investig* 2011;**1**:64-74.
4. Cui Z, Mumper RJ. Genetic immunization using nanoparticles engineered from microemulsion precursors. *Pharm Res* 2002;**19**(7): 939-46.
5. Wendorf J, Chesko J, Kazzaz J, Ugozzoli M, Vajdy M, O'Hagan D, et al. A comparison of anionic nanoparticles and microparticles as vaccine delivery systems. *Hum Vaccin* 2008;**4**(1):44-9.
6. Petros RA, DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. *Nat Rev Drug Disc* 2010;**9**:615-27.
7. Rolland JP, Maynor BW, Euliss LE, Exner AE, Denison GM, DeSimone JM. Direct fabrication and harvesting of monodisperse shape-specific nanobiomaterials. *J Am Chem Soc* 2005;**127**:10096-100.
8. Heurtault B, Saulnier P, Pech B, Proust JE, Benoit JP. Physico-chemical stability of colloidal lipid particles. *Biomaterials* 2003;**24**:4283-300.
9. Rolland JP, Van Dam RM, Schorzman DA, Quake SR, DeSimone JM. Solvent-resistant photocurable "liquid teflon" for microfluidic device fabrication. *J Am Chem Soc* 2004;**126**:2322-3.
10. Euliss LE, DuPont JA, Gratton SEA, DeSimone JM. Imparting size, shape and composition control of materials for nanomedicine. *Chem Soc Rev* 2006;**35**:1095-104.
11. Petros RA, Ropp PA, Desimone JM. Reductively labile PRINT™ particles for the delivery of dox to HeLa cells in vitro. *J Am Chem Soc* 2008;**130**:5008-9.
12. Kelly JY, DeSimone JM. Shape-specific monodisperse nano-molding of protein particles. *J Am Chem Soc* 2008;**130**:5438-9.
13. Napier ME, DeSimone JM. Nanoparticle drug delivery platform. *Polymer Reviews* 2007;**47**:321-7.
14. Gratton SEA, Williams SS, Napier ME, Pohlhaus PD, Zhou Z, Wiles KB, et al. The pursuit of a scalable nanofabrication platform for use in material and life science. *Acc Chem Res* 2008;**41**:1685-95.
15. Perry JL, Herlihy KP, Napier ME, DeSimone JM. PRINT: A novel platform toward shape and size specific nanoparticle theranostics. *Acc Chem Res* 2011;**44**(10):990-8.
16. Aguilar JC, Rodriguez EG. Vaccine adjuvants revisited. *Vaccine* 2007;**25**:3752-62.
17. Singh M, O'Hagan D. Advances in vaccine adjuvants. *Nat Biotechnol* 1999;**17**:1075-81.
18. Wack A, Baudner BC, Hilbert AK, Manini I, Nuti S, Tavarini S, et al. Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice. *Vaccine* 2008;**26**:552-61.

19. Subbarao K, Murphy BR, Fauci AS. Development of effective vaccines against pandemic influenza. *Immunity* 2006;**24**:5-9.
20. Horimoto T, Kawaoka Y. Designing vaccines for pandemic influenza. *Curr Top Microbiol Immunol* 2009;**333**:165-76.
21. Jeong W, Napier ME, DeSimone JM. Challenging nature's monopoly on the creation of well-defined nanoparticles. *Nanomedicine* 2010;**5**(4): 633-9.
22. Canelas DA, Herlihy KP, DeSimone JM. Top-down particle fabrication: control of size and shape for diagnostic imaging and drug delivery. *Nanomed Nanobiotechnol* 2009;**1**:391-404.
23. Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council. *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press; 1996.
24. Harlow E, Lane D. *Antibodies – A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY; 1988.
25. WHO Manual on Animal Influenza Diagnosis and Surveillance. http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf (website retrieved on 05 Jan 2012).
26. Welch BL. The significance of difference between two means when the population variances are unequal. *Biometrika* 1937;**29**: 350-62.
27. Milliken GA, Johnson DE. *Analysis of messy data*. London: Chapman and Hall; 1992.
28. Dunnett CW. A multiple comparison procedure for comparing several treatments with a control. *J Am Stat Assoc* 1955;**50**:1096-121.
29. Agresti A. *Categorical data analysis*. 2nd ed. New York: John Wiley and Sons; 2002. p. 211-66.
30. Uhrich KE, Cannizzaro SM, Langer RS, Shakesheff KM. Polymeric systems for controlled drug release. *Chem Rev* 1999;**99**:3181-98.
31. Jain JP, Ayen WY, Domb AJ, Kumar N. Biodegradable polymers in drug delivery. In: Domb AJ, Kumar N, Ezra A, editors. *Biodegradable Polymers in Clinical Use and Clinical Development*. Hoboken, NJ: John Wiley and Sons; 2011. p. 3-58.
32. Lim TY, Poh CK, Wang W. Poly (lactic-co-glycolic acid) as a controlled release delivery device. *J Mater Sci Mater Med* 2009;**20**(8):1669-75.
33. Sinha VR, Trehan A. Biodegradable microspheres for parenteral delivery. *Crit Rev Ther Drug Carrier Syst* 2005;**22**(6):535-602.
34. Stewart MJ, Plautz GE, Del Buono L, Yang ZY, Xu L, Gao X, et al. Gene transfer in vivo with DNA-liposome complexes: safety and acute toxicity in mice. *Hum Gene Ther* 1992;**3**:267-75.
35. San H, Yang Z-Y, Pmopili VJ, Jaffe ML, Plautz GE, Xu L, et al. Safety and short-term toxicity of a novel cationic lipid formulation for human gene therapy. *Hum Gene Ther* 1993;**4**:781-8.
36. Nabel GJ, Nabel EG, Yang Z-Y, Fox BA, Plautz GE, Gao X, et al. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. *Proc Natl Acad Sci USA* 1993;**90**:11307-11.
37. Pulendran B, Ahmed R. Immunological mechanisms of vaccination. *Nat Immun* 2011;**12**(6):509-17.
38. Davenport FM, Hennessy AV, Askin FB. Lack of adjuvant effect of AIPO, on purified influenza hemagglutinin in man. *J Immunol* 1968; **100**:1139-40.
39. Oyewumi MO, Kumar A, Cui Z. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune response. *Expert Rev Vaccines* 2010;**9**(9):1095-107.
40. Squalene-based adjuvants in vaccines. http://www.who.int/vaccine_safety/topics/adjuvants/squalene/questions_and_answers/en/ (website retrieved on 05 Jan 2012).