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Retention of structure, antigenicity, and biological function of pneumococcal surface protein A (PspA) released from polyanhydride nanoparticles

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Abstract

Pneumococcal surface protein A (PspA) is a choline-binding protein which is a virulence factor found on the surface of all *Streptococcus pneumoniae* strains. Vaccination with PspA has been shown to be protective against a lethal challenge with *S. pneumoniae*, making it a promising immunogen for use in vaccines. Herein, the design of a PspA-based subunit vaccine using polyanhydride nanoparticles as a delivery platform is described. Nanoparticles based on sebacic acid (SA), 1,6-bis-(*p*-carboxyphenoxy)hexane (CPH) and 1,8-bis-(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), specifically 50:50 CPTEG:CPH and 20:80 CPH:SA, were used to encapsulate and release PspA. The protein released from the nanoparticle formulations retained its primary and secondary structure as well as its antigenicity. The released PspA was also biologically functional based on its ability to bind to apolactoferrin and prevent its bactericidal activity towards *Escherichia coli*. When the PspA nanoparticle formulations were administered subcutaneously to mice, the animals elicited a high titer and high avidity anti-PspA antibody response. Together, these studies provide a framework for the rational design of a vaccine against *S. pneumoniae* based on polyanhydride nanoparticles.

Keywords

Pneumococcal surface protein A; polyanhydride; nanoparticle; *Streptococcus pneumoniae*; vaccine

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1. Introduction

The World Health Organization estimates that pneumonia causes 1.6 million deaths every year, with the majority occurring in children in developing countries [1, 2]. *Streptococcus pneumoniae* is the leading cause of bacterial pneumonia worldwide, especially in children [3]. Current prophylactic options against *S. pneumoniae* include a 23-valent soluble polysaccharide vaccine (pneumovax) and 7 and 13-valent pneumococcal conjugate vaccine (PCV) [4]. The polysaccharide-based vaccine has been shown to induce humoral immunity in immune-competent patients, but fails to stimulate a cellular immune response, making the vaccine ineffective in high-risk groups such as infants, the elderly, and immune-compromised individuals [5]. Following the introduction of the multivalent, polysaccharide-protein conjugate pneumococcal vaccine into childhood immunization regimens, incidence of community-acquired pneumonia in children was reduced by 18% [6]. Although the PCV has been shown to be effective in reducing cases of pneumonia, it has several limitations. The vaccine is expensive and complicated to manufacture, leading to limited availability in developing countries; it does not provide cross-protection across pneumococcal serotypes; and while it reduces capsular type-specific carriage it has not been shown to reduce nasopharyngeal carriage of pneumococci in general. Additionally, the PCV requires a three-dose vaccination regimen and the 7-valent vaccine leads to increased prevalence within the community of strains not included in the vaccine (i.e., serotype substitution) within several years of introduction [6, 7].

Subunit vaccines against pneumonia using non-capsular antigens, specifically protein-based vaccines, have been extensively studied in recent years [5,8]. Of particular interest in this regard is pneumococcal surface protein A (PspA), which is a choline-binding protein found on the surface of all pneumococcal strains and a critical *S. pneumoniae* virulence factor [9]. PspA plays two different roles in invasive infection and nasopharyngeal carriage. During invasive, systemic infections with *S. pneumoniae*, PspA prevents the deposition of complement on the surface of the bacterium, thus inhibiting the opsonization and killing of *S. pneumoniae* [9, 10]. PspA also inhibits bactericidal activity medicated by apolactoferrin (ALF) found on mucosal surfaces and in sites of inflammation [9, 11–13]. Vaccination with PspA protects mice against a lethal challenge with *S. pneumoniae* via the generation of anti-PspA serum antibodies that are highly cross reactive to other strains [14–18]. However, PspA is poorly immunogenic and not capable of inducing a productive immune response without the addition of an adjuvant [19–21]. In fact, a vaccine regimen based on the inclusion of aluminum hydroxide, a commonly used adjuvant, required three doses to provide protective immunity in a murine model [19–21]. Therefore, there is a need to design novel adjuvants and/or delivery vehicles for the formulation of efficacious vaccines that can protect against multiple strains of *S. pneumonia* and enhance patient compliance by utilizing an acceptable dose regimen.

Because of the promise of PspA as a protective antigen against *S. pneumoniae*, it has been the subject of numerous studies to evaluate novel vaccine delivery systems. Several research groups have shown the induction of immune responses through delivery of PspA with live attenuated bacteria such as salmonella [22,23] and through co-delivery with a whole-cell pertussis vaccine [4]. Additionally, other novel, nanoscale delivery systems containing PspA have been evaluated, including gold nanoparticles [24] and nanogel-based vaccine formulations [25]. In this work, we demonstrate that biodegradable polyanhydride nanoparticles can successfully encapsulate and release stable, antigenic PspA.

Polyanhydrides have a number of benefits compared to other vaccine delivery systems. Their tunable polymer chemistry can allow for the modulation of the immune response and enable tailoring antigen release kinetics [26, 27]. Additionally, encapsulation into

polyanhydride particles has been shown to protect fragile protein antigens from degradation [28, 29]. Polyanhydrides can be fabricated into nanoparticles for administration via inhalation or injection and have shown much promise as vaccine adjuvants and delivery vehicles [26, 30–33]. These polymers exhibit excellent biocompatibility and have been shown to degrade into non-toxic, non-mutagenic products [34]. Polyanhydride particles have also been shown to stabilize fragile proteins throughout the manufacture, storage, and release steps and elicit immune responses *in vitro* and *in vivo* [28–30, 32, 35, 36]. In particular, amphiphilic polyanhydrides, which degrade through a combination of bulk and surface erosion, provide a sustained release of protein, while maintaining protein structure and function upon release [28, 37]. For example, encapsulation of the recombinant F1-V protein into nanoparticles made from a copolymer of 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) and 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) stabilized the protein [28] and provided protective immunity against a lethal challenge with *Yersinia pestis* that persisted at least 23 weeks post-immunization using a single dose vaccine regimen [32].

In this work, we describe the encapsulation and release of stable PspA from polyanhydride nanoparticles. The released PspA retained its primary and secondary structure and preserved both its antigenicity and biological functionality. When the nanoparticle-based vaccine formulations consisting of soluble and encapsulated PspA were administered subcutaneously to mice, the animals developed and sustained high anti-PspA IgG titers that were also characterized by high avidity. These studies provide a framework for the rational design of an anti-*S. pneumoniae* vaccine based on PspA-containing polyanhydride nanoparticles.

2. Materials and Methods

2.1 Materials

The materials used for monomer synthesis including sodium hydroxide, hydrobenzoic acid, dibromohexane, 1-methyl-2-pyrrolidinone, and triethylene glycol were purchased from Sigma Aldrich (St. Louis, MO); acetone, sulfuric acid, potassium carbonate, dimethyl formamide, toluene, acetonitrile, N,N-dimethylacetamide, and acetic acid were purchased from Fisher Scientific (Fairlawn, NJ); and 4-*p*-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK). Sebacic acid monomer was purchased from Sigma Aldrich. The following chemicals used for acetylation and polymerization were purchased from Fisher Scientific: acetic anhydride, ethyl ether, petroleum ether, chloroform, methylene chloride, and hexane. Deuterated chloroform and dimethyl sulfoxide for ^{1}H NMR analysis of the polymers and monomers, respectively, were purchased Cambridge Isotope Laboratories (Andover, MA).

The K-12 MG1655 and the LPS-deficient K-12 NR688 *Escherichia coli* strains were provided by Dr. Gregory Phillips (Iowa State University).

The following chemicals used in the development of the PspA functional assay were purchased from Sigma Aldrich: ALF, lactoferricin, erythromycin, deferoxamine, and 2,2-bipyridine. The following materials for bacterial culture were purchased from Becton Dickinson (Franklin Lakes, NJ): Luria-Bertani (LB) agar, trypticase soy broth, yeast extract, and tryptone. Agar and sodium chloride were purchased from Fisher Scientific. Sterile phosphate-buffered saline was purchased from Mediatech Inc. (Manassas, VA).

2.2 Polymer synthesis

The CPH and CPTEG monomers and their corresponding prepolymers were synthesized as previously described [37–39]. Polymers and copolymers of CPH, CPTEG and SA were synthesized using melt condensation as described elsewhere [34, 37]. Polymer purity and molecular weight were determined using ^{1}H NMR (Varian VXR300) and gel permeation

chromatography (Waters GPC, Milford, MA) was used to confirm molecular weight. The purity and molecular weight of the 50:50 CPTEG:CPH and 20:80 CPH:SA copolymers used herein were consistent with previously published data [37, 40]. The copolymer compositions were determined by ^{1}H NMR and were consistent with the co-monomer compositions (i.e., 18:82 for 20:80 CPH:SA and 47:53 for 50:50 CPTEG:CPH). The number-average molecular weights of the 50:50 CPTEG:CPH and 20:80 CPH:SA copolymers were 5,800 Da with a polydispersity index (PDI) of 1.5 and 21,000 Da with a PDI of 1.4, respectively.

2.3 Nanoparticle fabrication

PspA-loaded polyanhydride nanoparticles were formulated using an anti-solvent, solid/oil/oil nanoprecipitation method as previously described [41–43]. Briefly, lyophilized PspA (1% w/w) and polymer (20 mg/mL) were co-dissolved in methylene chloride. The solution was sonicated at an output of 40 Hz with a VibraCell ultrasonic probe (Sonics & Materials, Inc., Newton, CT) to ensure a homogenized mixture. The resulting solution was rapidly added to pentane at a 1 to 250 (v/v) ratio of methylene chloride to pentane at room temperature for the CPH:SA formulation or at −40°C for the CPTEG:CPH copolymer due to the lower glass transition temperature of this polymer [37]. Nanoparticles were collected using vacuum filtration. The nanoparticles were characterized using scanning electron microscopy (FEI Quanta SEM, Hillsboro, OR) and their size distribution was determined using ImageJ software (Version 1.44p, National Institutes of Health, Bethesda, MD). The resulting size distributions were 677 ± 254 nm for the 20:80 CPH:SA nanoparticles and 243 ± 84 nm for the 50:50 CPTEG:CPH nanoparticles.

2.4 Preparation of recombinant protein

The N-terminal region of a recombinant PspA (UAB055, PspA/Rx1 AA1 to 303, clade 2 PspA of the PspA family 1) was produced by Dr. David McPherson (University of Alabama at Birmingham) as described previously [12]. Protein was dialyzed using a 10,000 MW cut-off dialysis cassette (Thermo Fisher Scientific, Rockford, IL) against sterile, nanopure water and frozen at −80°C. Protein was then lyophilized at −40°C under vacuum overnight and stored at −80°C until further use. For the *in vivo* vaccination and biological functionality assays, endotoxin was removed from the protein using endotoxin removal beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions prior to dialysis and lyophilization. The final endotoxin content of the protein prior to use was determined to be less than 1.9 EU/mg as determined by a limulus amebocyte lysate (LAL) chromogenic endotoxin quantification kit (Thermo Fisher Scientific).

2.5 Culture and storage of bacterial strains

Two *E. coli* strains were used in this work, wild type MG1655 and LPS-deficient NR688. Prior to the start of experiments, LB broth was inoculated with approximately one colony of *E. coli* and allowed to grow overnight. Fresh LB broth was inoculated and incubated until an OD_{600} of approximately 0.7 was obtained. Sterile glycerol (15% v/v) was added to aliquots of the bacterial culture and 1 mL aliquots were frozen and stored at −80°C.

2.6 Protein release and quantification

The *In vitro* release of PspA protein from polyanhydride nanoparticles was carried out as previously described [28, 30, 35, 37, 44]. Briefly, 25 mg of nanoparticles loaded with 1% (w/w) PspA were weighed into 1.5 mL microcentrifuge tubes and suspended in 600 μL of 0.05 M phosphate buffered saline (PBS, pH 7.2). Microcentrifuge tubes containing the suspensions were incubated at 37°C under constant agitation. At each time point, suspensions were removed and centrifuged at 14,000 rcf for 20 min. The supernatant was removed and replaced with 450 μL of fresh PBS and the microcentrifuge tubes returned to

incubation. Supernatant samples were stored at 4°C until analyzed. Supernatant samples were centrifuged at 10,000 rcf for 10 min before protein quantification to ensure removal of any remaining polymer particles. Protein released from polyanhydride nanoparticles was quantified using a micro bicinchoninic acid assay (Thermo Fisher Scientific) and was compared to the total protein used. The colorimetric changes were measured using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA) at an absorbance of 570 nm. All the samples were tested in triplicate. Encapsulation efficiency was determined by degrading PspA-loaded particles in a solution of 17 mM NaOH. In order to determine the pH of the particle microenvironment over time 25 μg of 20:80 CPH:SA and 50:50 CPTEG:CPH nanoparticles were incubated in 600 μL of 0.05 M phosphate buffered saline (PBS, pH 7.2). At each time point samples were centrifuged and the supernatant was removed for analysis. Solution pH was measured using an Orion Ross pH electrode (Thermo Fisher Scientific).

2.7 Analysis of released PspA by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The primary structure of the released PspA was studied using SDS-PAGE under reducing conditions. After centrifugation, the protein concentration was adjusted to 100 μg/mL with PBS. Once concentrations of all the samples were standardized, 15 μL of solution was combined with 15 μL of Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% (v/v) β-mercaptoethanol (Bio-Rad). The resultant mixture was heated at 96°C for 10 min and allowed to return to room temperature. Samples were loaded into the wells of a 12% acrylamide gel and subjected to 150 V constant voltage for 60 min. Approximate molecular size of protein bands were estimated using pre-stained protein standards (Bio-Rad, Hercules, CA). Gels were incubated in fixative solution (50% water, 40% ethanol, 10% acetic acid) for 3 h at 4 °C and stained with Flamingo Gel Stain (Bio-Rad) at 4°C overnight before scanning. Gels were scanned with a Typhoon 9400 (GE Healthcare Piscataway, NJ) and analyzed using ImageQuant TL 8.1 software (GE Healthcare).

2.8 Secondary structure of released PspA

Circular dichroism was used to analyze the secondary structure of PspA released from polyanhydride nanoparticles. Aliquots of released PspA (100 μg/mL), collected after incubating for two hours in PBS at 37°C, were analyzed on a Jasco J-710 spectropolarimeter (Jasco, Inc., Easton, MD) using a 1 mm cuvette at far-UV wavelengths from 190–260 nm. Values shown are the average of two readings of at least two experimental replicates. As a control, to investigate the degradation of PspA under acidic conditions, 100 μg/mL of PspA was incubated with nanopure water adjusted to a pH of 2.0 with 12.1 N HCl (Thermo Fisher Scientific) and secondary structure was analyzed as described above.

2.9 Antigenicity of released protein

The ability of anti-PspA antibody to bind to PspA released from polyanhydride nanoparticles (i.e., antigenicity) was determined using an enzyme-linked immunosorbent assay (ELISA) as previously described [28, 29]. PspA released from polyanhydride nanoparticles was collected, adjusted to a concentration of 0.5 μg/mL, used to coat a high-binding chemistry 96-well plate (Cole-Parmer, Vernon Hills, IL), and incubated overnight at 4°C. The PspA solution was removed and PBS with 1% fish gelatin was added and incubated for 2 h at room temperature. Next, the blocking buffer was removed and the plates were washed three times with PBS containing 0.5% Tween 20 (PBS-T). Anti-PspA monoclonal antibody clone number 22003 (QED Biosciences, San Diego, CA) was added at a concentration of 1 μg/mL and the plates were covered and incubated overnight at 4°C. Plates were washed thrice with PBS-T before the addition of alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) (Jackson ImmunoResearch, West

Grove, PA) at a 0.1 μg/mL, which was incubated for 2 h at room temperature before developing. For the development of the ELISA, alkaline phosphatase substrate was added at 1 mg/mL substrate buffer (50 mM sodium carbonate 2 mM magnesium chloride buffer (pH 9.3)). After a 15 min incubation at room temperature, the colorimetric changes were measured at an absorbance of 405 nm using a SpectraMax M3 microplate reader. The data is presented as relative antigenicity, which is herein defined as the optical density (OD) ratio between the experimental replicate and the native protein.

2.10 Functional assay for released PspA

E. coli strains NR688 (LPS-deficient) and wild type MG1655 (used as an experimental control), were cultured on Luria-Bertani (L-B) agar plates at 37°C overnight. Approximately one colony was removed from the overnight plate and used to inoculate broth. It was necessary to remove iron from the experimental system in order to prevent binding to ALF, which forms iron-rich hemolactoferrin. To accomplish this, both iron-free media and the iron-chelating agent, deferoxamine, were tested, and it was found that the addition of 1 mg/mL of deferoxamine successfully removed iron. The inoculated culture was grown at 37°C to an OD_{600} of approximately 0.5. Cultures were diluted 1:20 into fresh broth and incubated until an OD_{600} of approximately 0.1 was obtained. Bacteria were plated into the wells of a 96-well microtiter plate at a final concentration of 1 million cells per well, as estimated by a SmartSpec 3000 (Bio-Rad). ALF was dissolved in the appropriate medium at 3 mg/mL and added to the plated bacteria. After incubation for 3 h at 37°C, 10 μL of the solution was removed and added to 90 μL of the appropriate broth. Six 1:10 dilutions were prepared before plating in triplicate on L-B agar plates. The plates were incubated at 37°C overnight and colony forming units (CFUs) were counted.

The same procedure was followed to test the abrogation of killing by ALF through the addition of recombinant PspA. Briefly, *E. coli* was grown on LB agar overnight, a colony was inoculated into 5 mL LB broth and incubated at 37°C until the culture reached its exponential growth phase. The culture was diluted 1:20 into fresh broth and grown to its early log growth phase before inoculating at 10^6 cells/well of a microtiter plate. ALF was dissolved in medium at a concentration of 3 mg/mL followed by the addition of native PspA or PspA collected after release from nanoparticles, which was mixed and allowed to incubate for approximately 5 min before addition to the bacteria. The microtiter plate was incubated at 37°C for 3 h before enumerating viable bacteria as described above. Although the nanoparticles were removed prior to the addition of released PspA, it was verified that the presence of nanoparticles did not interfere with the experimental system. Additionally, it was confirmed that the acidic microenvironment produced by the nanoparticle degradation products did not interfere with the growth of *E. coli*.

CFUs were determined by counting colonies grown on LB agar plates after overnight incubation. The log CFU killed was calculated by subtracting the experimental values from control *E. coli* cultures grown under identical conditions. Data is presented as log CFU of killed *E. coli*.

2.11 Immunization studies

CBA/CaHN-Btk^{xid}/J (CBA/N) mice purchased from Jackson Laboratory (Bar Harbor, ME) were used for the immunization studies. Mice were immunized subcutaneously with 20 to 25 μg reconstituted PspA and 0 to 5 μg PspA encapsulated into 500 μg of polyanhydride nanoparticles and/or formulated with MPLA (10 μg, derived from *Salmonella enterica* serotype Minnesota Re 595 (Sigma Aldrich)), a traditional adjuvant approved for human use [45]. The treatment groups were: 20 μg PspA delivered solubly with 5 μg PspA encapsulated into 500 μg of 50:50 CPTEG:CPH nanoparticles, 20 μg PspA delivered

solubly with 5 μg PspA encapsulated into 500 μg of 20:80 CPH:SA nanoparticles, 25 μg PspA delivered solubly with 10 μg MPLA, and a saline control. The volume of the inoculum was 100 μL in each case. At least six animals were used in each experimental group. Blood was collected from animals via the saphenous vein before immunization and at 2, 4, and 7 weeks post-immunization. Serum was collected after centrifugation (10,000 rcf for 10 min) of the blood samples and stored at 4°C until use.

2.12 Anti-PspA serum antibody titers

Antibody titers were determined using an ELISA as described above. Plates were coated with PspA at a concentration of 0.5 μg/mL and incubated overnight at 4°C. The blocking buffer used for these studies was 0.05 M PBS-T supplemented with 2% gelatin (Becton Dickinson, Franklin Lakes, NJ). PspA coated microtiter plates were incubated for 2 h at room temperature before washing thrice with PBS-T. Serum from immunized mice was added to the first well at a 1:200 dilution in PBS with 1% (v/v) heat-inactivated goat serum and serially diluted two-fold. After an overnight incubation at 4°C, plates were washed three times with PBS-T, and then alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) (Jackson ImmunoResearch) was added at a concentration of 1 μg/mL followed by incubation for 2 h at room temperature. Plates were washed four times with PBS-T and 1 mg/mL alkaline phosphatase substrate (Fisher Scientific, Pittsburgh, PA) dissolved in 50 mM sodium carbonate 2 mM magnesium chloride buffer (pH 9.3) was added for colormetric development. The plates were read after 30 minutes at 405 nm. All the samples were tested in technical replicates of 2. Titer is herein defined as the serum dilution that produced an optical density value twice that of the saline group.

2.13 Antibody avidity

MagPlex-C microspheres (magbeads) were activated and coupled to PspA (20 μg/mL in PBS) as described by the manufacturer. Following the conjugation step, the PspA-magbeads were washed with PBS-T and stored in PBS with 1% bovine serum albumin and 0.05% sodium azide and covered to block overnight at 4°C. Serum samples were diluted in PBS-T and 50 μL was added to each well of a 96 well black, clear bottom microtiter plate along with 50 μL of vigorously vortexed magbeads (120,000/mL) conjugated with PspA suspended in blocking buffer. Plates were immediately covered and placed on a plate shaker for 1 h at room temperature. Plates were washed three times with the blocking buffer using a BioPlex Pro II plate washer and 100 μL of either PBS-T or 6 M urea in PBS (chaotriopic agent) were added to the appropriate wells. Plates were covered and put on a shaker for 15 min, and then washed three more times. Biotin-conjugated goat anti-mouse IgG (eBioscience, San Diego, CA) was diluted 1:200 and 50 μL was added to each well, and plates were covered and shaken at room temperature for 1 h. Plates were washed three times with wash buffer, 50 μL of streptavidin-phycoerythrin (PE) diluted 1:20 in PBS-T was added to each well, and incubated for 30 min. Plates were washed three times and filled with 100 μL of blocking buffer per well, and the mean fluorescent intensity (MFI) was measured using a BioPlex 200. To calculate the relative avidity of a serum sample having a demonstrable antibody response to the target antigen, the MFI obtained for the sample well treated with 6 M urea was divided by the MFI obtained for the corresponding sample well incubated with only PBS-T. This value was multiplied by 100 to obtain a relative percent avidity for each serum sample.

2.14 Calculations and statistics

Log CFU killed were calculated by subtracting the log of the experimental group CFU/mL concentration from the log CFU/mL of bacteria alone after incubation. All error bars represent standard error of the mean (S.E.M). MiniTab statistical package (MiniTab, Inc.,

State College, PA) was used to perform statistical analysis. A two sample t-test was used and statistical significance was characterized by $p < 0.05$.

3. Results and Discussion

Several proteins, including F1-V, *Bacillus anthracis* protective antigen (PA), bovine serum albumin (BSA), and ovalbumin have been shown to be stably released from polyanhydride particles [28, 29, 30, 36]. Each of these proteins has different mechanisms of instability and/or degradation. Herein, the lessons learned from these previous studies were applied to determine the optimal polyanhydride nanoparticle formulations for stabilization and sustained delivery of functional PspA for the development of a vaccine against *S. pneumoniae*.

3.1 Polyanhydride nanoparticles provide for the release of stable and functional PspA

PspA was encapsulated into and released from 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles to study the release kinetics of the protein from different polyanhydride chemistries. These chemistries were chosen based on the previous success of 50:50 CPTEG:CPH-based nanovaccine formulations in inducing protective immunity [32] and the ability of 20:80 CPH:SA to stabilize proteins for extended periods at elevated temperatures, obviating the need for cold storage [29]. Additionally, 20:80 CPH:SA nanoparticles are readily internalized by antigen presenting cells, which could lead to enhanced internalization of PspA by these immune cells [41]. The particles were fabricated using a solid/oil/oil double emulsion method with encapsulation efficiencies of 62 and 68% for 50:50 CPTEG:CPH and 20:80 CPH:SA, respectively. PspA-loaded nanoparticles were characterized using SEM and the photomicrographs are shown in Figure 1. The size and shape of the nanoparticles were consistent with previous work using polyanhydride nanoparticles, with smooth, roughly spherical surface morphology and an average diameter of 677 ± 254 nm for the 20:80 CPH:SA nanoparticles and 243 ± 84 nm for the 50:50 CPTEG:CPH particles [41, 42]. Particles were stored at −20°C until use and thoroughly sonicated before administration. Figure 2 shows the release of PspA from polyanhydride nanoparticles over 30 days. Protein release from the 20:80 CPH:SA and 50:50 CPTEG:CPH chemistries showed an initial burst of 36% and 45%, respectively, followed by a sustained release, with smaller amounts of protein released from the particles over a period of time. The near-zero order release profile (after the initial burst) can be attributed to the surface erosion characteristics of polyanhydrides, in contrast to the bulk erosion exhibited by PLGA and other biodegradable polymers [46, 47]. The burst release observed is due to the relatively higher amount of surface area of the particle at initial time points and because of the propensity of the payload to migrate towards the surface of the particle during solvent evaporation. This burst release provides sufficient antigen initially to prime the immune response. These data are consistent with previous work on protein release kinetics from polyanhydride particles [28, 29, 35–37].

3.2 Preservation of PspA primary and secondary structure upon release from polyanhydride nanoparticles

Following the release of PspA from the nanoparticles, the supernatants were collected and the primary and secondary structures were analyzed using SDS-PAGE and circular dichroism (CD), respectively. Figure 3 shows that the primary structure of PspA remained intact after encapsulation and release based on the presence of a single band just under 50 kDa and the absence of smaller bands in all the lanes of the gel. Additionally, the lack of any larger bands indicated that the polyanhydride nanoparticle formulations did not cause any PspA aggregation.

It is known that the N-terminal portion of the recombinant PspA used in this work adopts a coiled coil, α-helical secondary structure [48]. Figure 4 shows CD spectra for PspA released from the different polyanhydride nanoparticle formulations. The data showed that the α-helical secondary structure of the protein was maintained after release from the nanoparticles, as evidence by the presence of characteristic minima at 208 nm and 222 nm, which are known to be associated with an α-helical structure [49]. Additionally, the observed secondary structure is consistent with previously published work on PspA [48]. Figure 4 also shows that PspA incubated under acidic conditions loses its secondary structure as evidenced by the absence of the characteristic minima at 208 nm and 222 nm.

It is significant that both polyanhydride chemistries tested preserved the structure of PspA upon release. The option to choose from multiple chemistries provides the ability to tailor both the protein release profile and the host immune response. Differences in adjuvant chemistry, especially with respect to hydrophobicity and degradation kinetics, have been shown to significantly influence the character of the resulting immune response. For example, the 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticle formulations differentially affect cytokine secretion [27]. These two polymer chemistries also have different *in vivo* distribution and inflammation profiles, which can be exploited to further modulate the desired immune response [50]. Moreover, 20:80 CPH:SA nanoparticles have excellent thermal properties and can preserve protein antigenicity for up to one year at room temperature [29]. This feature could be exploited to eliminate the requirement for maintaining a cold chain during vaccine storage and delivery [51].

3.3 Antigenicity of released PspA is retained upon release from polyanhydride nanoparticles

The results depicted in Figure 5 show that the antigenicity of PspA is maintained after release from the polyanhydride nanoparticles. The antigenicity was determined using an ELISA with a PspA-specific monoclonal antibody; this assay measured the ability of PspA released from polyanhydride nanoparticles be recognized by an anti-PspA-specific antibody. The relative antigenicity of the PspA released from nanoparticles was preserved for both polyanhydride chemistries tested. These results indicated that PspA may not be as susceptible to hydrophobicity-induced conformational changes that have been observed with other proteins [28]. However, it was observed that incubating PspA in acidic solutions, pH 5 and below, led to protein degradation (data not shown). Many biodegradable polymers, including polyanhydrides and polyesters such as PLGA, have acidic degradation products and, as demonstrated for PspA, incubation in acidic solution can lead to protein degradation. The amphiphilic 50:50 CPTEG:CPH has been shown to have local microenvironments of approximately pH 6.0, as compared to more acidic chemistries such as 20:80 CPH:SA and PLGA, which have been shown to have local microenvironments of pH < 4.0 [37]. Supplemental Figure 1 shows the change in the pH of the release buffer in which the polyanhydride nanoparticles are eroding over time. These data show that degradation of 50:50 CPTEG:CPH nanoparticles results in a small decrease in pH, with a stable pH of 6.9 maintained for the duration of the study. In contrast, the degradation of the 20:80 CPH:SA nanoparticles resulted in a more pronounced pH decline to pH~5.2. Given this information, it is expected that PspA released from 50:50 CPTEG:CPH nanoparticles would maintain antigenicity, while PspA released from 20:80 CPH:SA nanoparticles would degrade from acidic effects. However, in the current experiments, PspA released from both 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles maintained 90% relative antigenicity. Unlike bulk-eroding polymers such as PLGA, polyanhydrides degrade through surface erosion, which may explain why the PspA was well preserved. This erosion characteristic protects the encapsulated protein from the acidic microenvironment by preventing water penetration into the bulk [46]. It is also important to note that during these *in vitro* release studies,

released protein remained under these acidic conditions for 24 h before the supernatant was removed for testing and replaced with fresh PBS. However, when administered *in vivo*, polyanhydride nanoparticle vaccine formulations disseminate quickly, which would prevent the local build-up of acidic degradation products [50]. The preservation of 90% relative antigenicity of PspA upon release from both the 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles under these harsh *in vitro* conditions indicates that these formulations may be promising delivery vehicles for *in vivo* administration.

3.4 Polyanhydride nanoparticles preserve the biological functionality of released PspA

In order to test the biological functionality of PspA released from polyanhydride nanoparticles, an *E. coli* killing assay was developed. This assay takes advantage of the bactericidal nature of ALF, which is found in the human mucosa in two forms, the iron-deficient ALF and the iron-rich hemolactoferrin [12]. The role of PspA in pneumococcal infection is to bind to ALF and prevent its bactericidal and bacteriostatic effects [12]. In the current studies, two *E. coli* strains were tested, the wild type MG1655 and the LPS-deficient NR688. Due to the absence of LPS, the NR688 strain proved to be more susceptible to killing by ALF.

Figure 6 shows that PspA released from both polyanhydride nanoparticle formulations retained its biological functionality using native PspA as a control. The log CFU of *E. coli* killed by ALF in the nanoparticle groups was statistically different from that of the untreated ALF group but not statistically different from the native PspA control. This indicates that the PspA protein released from polyanhydride nanoparticles is able to perform its biological function as well as the PspA control.

3.5 Vaccination with a single dose of PspA-containing polyanhydride nanoparticles provides a sustained immune response

CBA/N mice were vaccinated subcutaneously with polyanhydride nanoparticle-based and MPLA-based single dose vaccination regimens. Each animal was given a total of 25 μg of PspA. For the MPLA group, 10 μg of MPLA was used as an adjuvant. For the nanoparticle groups, 20 μg of PspA was delivered as soluble protein along with 5 μg of PspA encapsulated into 500 μg of polyanhydride nanoparticles of the specified chemistry. Based on the results from the *in vitro* studies described above, both nanoparticle formulations were tested. All vaccinated mice, regardless of chemistry (except for the saline formulation), developed anti-PspA antibody titers between 8,000 and 20,000 by seven weeks post-immunization (Figure 7a). These titers are consistent with those reported to be protective against lethal challenge with *S. pneumoniae* [15]. The mice receiving the 50:50 CPTEG:CPH nanoparticle vaccine formulation maintained their titer for at least seven weeks at titers above 10,000. The mice administered the 20:80 CPH:SA nanoparticle formulation and the MPLA formulation tended to have a decline in titer between weeks four and seven, although not statistically significant. In other work, the controlled release provided by nanoparticle-based vaccine formulations, specifically the 50:50 CPTEG:CPH chemistry, has sustained titer 23 weeks post-immunization and provided long-lasting immunity in a single dose [32]. The prolonged presence of protein as a consequence of the persistence of the nanoparticles may explain the effectiveness of the soluble plus encapsulated protein combination in the nanoparticle-based vaccine formulations.

The sustained release of protein is important as the continuous release of small quantities of antigen may be significant for the induction of a long-lasting immunity characterized by a high titer, high avidity antibody response. This effect may also have important implications in the development of a single dose PspA vaccine, limiting the need for repeat administrations. Many vaccine regimens typically require more than one immunization,

including the current *S. pneumoniae* 7 and 23-valent conjugate vaccines, which require three primary doses in infants and a booster dose at age two [52]. The single dose administration of particles based on biodegradable polymers such as polyanhydrides has demonstrated the ability to provide antibody titers consistent with those induced by multiple doses of traditional adjuvants [52]. Continuous release of antigen would mimic a replicating pathogenic infectious agent and generates a robust, long-lasting immune response as the continual exposure of B cells to antigen induces a strong memory response [53]. Additionally, the initial burst of released antigen observed with both the 20:80 CPH:SA and 50:50 CPTEG:CPH nanoparticle formulations may provide sufficient antigen to successfully prime the immune response and generate T cell help. This step is critical because T cell interactions with B cells lead to development of germinal centers and generation of high affinity antibodies [54].

To assess the quality of the antibody response, avidity was measured. High avidity is indicative of high strength of binding antibody, which is important for a sustained and effective humoral immune response [55]. The data in Figure 7b shows the relative avidity of the serum anti-PspA IgG antibody response at seven weeks post-immunization. Both of the polyanhydride chemistries tested demonstrated similar relative avidities, with the 50:50 CPTEG:CPH nanoparticle formulation resulting in a slightly higher relative avidity, though not significantly so, over the 20:80 CPH:SA. Additionally, the variability of the avidity of the anti-PspA antibody between mice was smaller with the nanoparticle formulations compared to that of the MPLA-immunized animals. The induction of highly avid antibodies by polyanhydride nanoparticle formulations is consistent with the sustained release demonstrated by these particles *in vitro*. The avidity indices observed in this work were similar to those generated against capsular polysaccharide 6B through a triple-immunization of an 11-valent pneumococcal conjugate vaccine adjuvanted with alum [56]. In that study, it was demonstrated that the addition of alum to the vaccine did not provide a benefit with respect to avidity. Those observations, paired with our present findings, highlight an important contrast between the use of alum and polyanhydride nanoparticle-based vaccine platforms in terms of the ability to increase antibody avidity [57].

This current work demonstrates the importance of rationally designing adjuvants and/or delivery vehicles for vaccine optimization. The encapsulation of PspA into polyanhydride nanoparticles provided a number of benefits over using off-the-shelf adjuvants such as MPLA, including sustained release, long-lasting immune responses, and higher avidity antibodies, as demonstrated previously with other protein antigens [32, 53]. Because PspA was found to be structurally stable, antigenic, and biologically functional upon release from two separate polyanhydride nanoparticle chemistries, there exists the ability to rationally select nanoparticle formulations to protect vaccine antigens while tailoring the immune response to a phenotype that is most effective against *S. pneumoniae*. The current studies lay the groundwork for the design of more robust and versatile vaccines against pneumonia that have the potential to provide significant benefits over current vaccination regimens.

5. Conclusions

The primary and secondary structure, antigenicity, and biological functionality of PspA protein were preserved during encapsulation and release from polyanhydride nanoparticles. This result is significant because the release of intact, functional protein increases the probability of preserving important, neutralizing epitopes and facilitates the development of an effective immune response. Herein, we demonstrate not only the release of functionally intact protein, but also demonstrate how a PspA vaccine based on polyanhydride nanoparticles can be used to generate a robust humoral immune response. PspA-containing polyanhydride nanoparticle vaccine formulations induced stable, high titers and avid

antigen-specific antibody. These studies provide a critical foundation for the future design of robust and versatile vaccines against streptococcal pneumonia that address both colonization and infection in a more patient-friendly manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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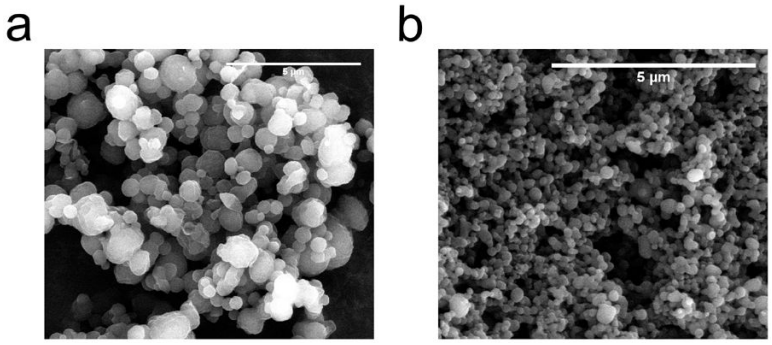

Figure 1.
Representative scanning electron photomicrographs of sonicated 1% PspA encapsulated (a) 20:80 CPH:SA and (b) 50:50 CPTEG:CPH nanoparticles. Scale bar: 5 μm.

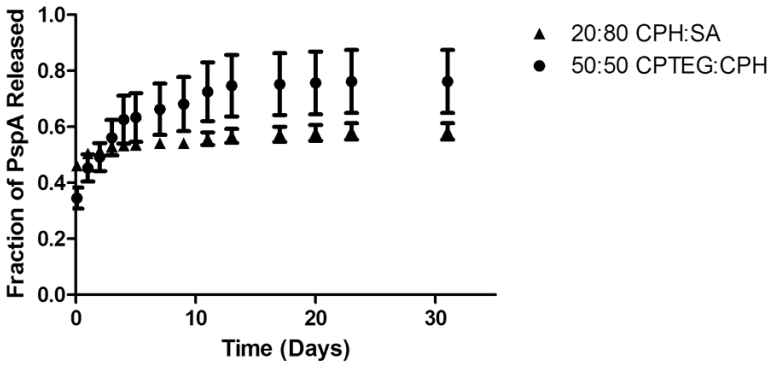

Figure 2.

Kinetic profile of PspA released from two polyanhydride nanoparticle formulations. Cumulative fraction of PspA released from 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles is presented as a function of time. Data are presented as the mean ± SEM of at least two independent experiments with three technical replicates each.

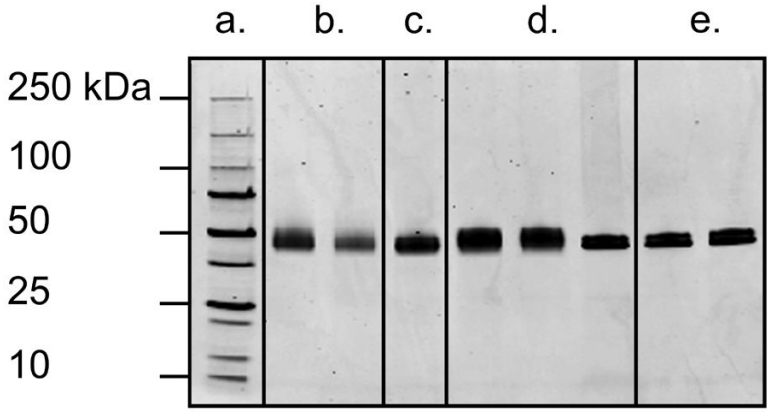

Figure 3.
The primary structure of PspA was preserved upon release from polyanhydride nanoparticles. Lanes: a) Molecular weight ladder, b) PspA, c) PspA plus MPLA, d) PspA released from 50:50 CPTEG:CPH nanoparticles, and e) PspA released from 20:80 CPH:SA nanoparticles.

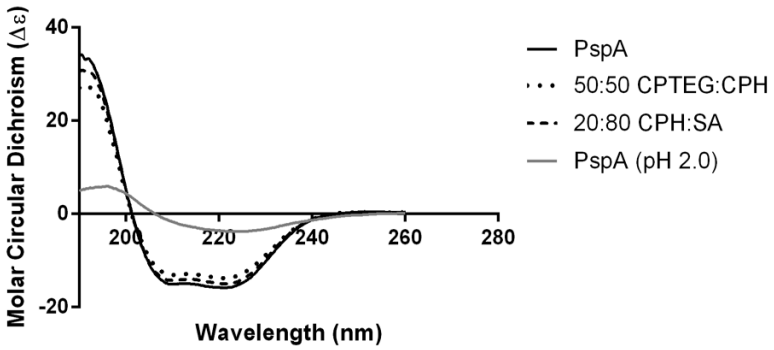

Figure 4.

The secondary structure of PspA was preserved during encapsulation and release from polyanhydride nanoparticles. The presence of characteristic minima at 208 and 222 nm indicates preservation of α-helical secondary structure. However, PspA incubated under acidic conditions loses its secondary structure. Each measurement represents the average of at least two independent experiments, with two technical replicates each.

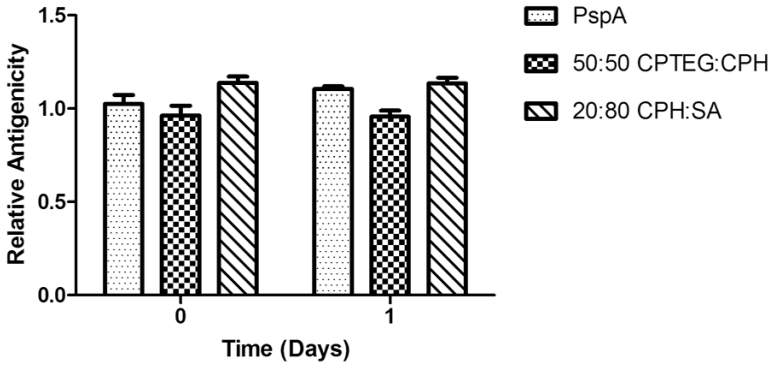

Figure 5.

Antigenicity of PspA is preserved upon release from polyanhydride nanoparticles. Relative antigenicity is defined as the optical density ratio between the experimental replicate and the native protein. Day 0 represents the antigenicity of released protein samples after two hours of incubation. Data are presented as the mean ± SEM of at least two independent experiments with three technical replicates each.

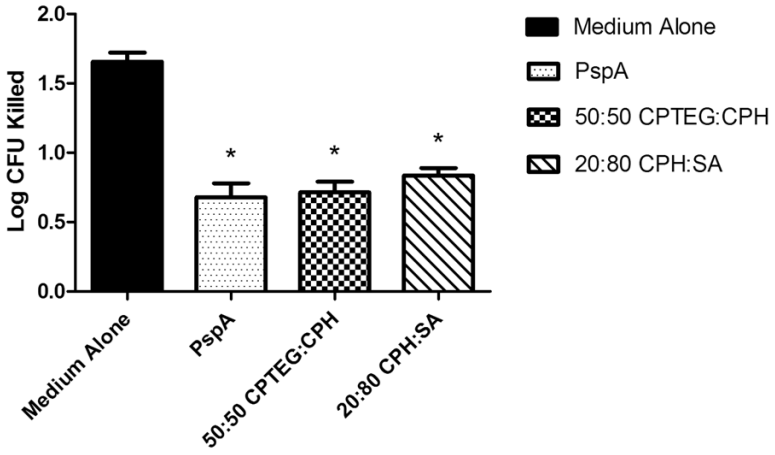

Figure 6.

PspA released from nanoparticles retained biological functionality as indicated by the ability to reduce the bactericidal activity of ALF. Separate cultures of *E. coli* K-12 NR688 were incubated with bacteria culture medium containing ALF alone, ALF plus control PspA, or ALF plus PspA released from either 50:50 CPTEG:CPH or 20:80 CPH:SA as described in Materials and Methods. Data are presented as the mean ± SEM of two independent experiments with at least six technical replicates each. Treatments identified with asterisks are statistically different from the cultures treated with ALF alone ($p < 0.005$).

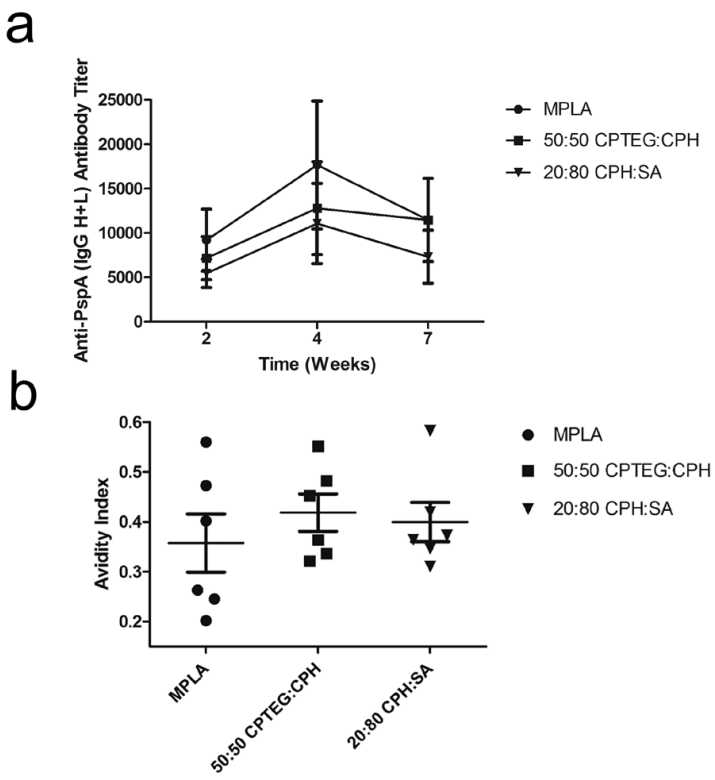

Figure 7.

CBA/N mice immunized with polyanhydride nanoparticle formulations produced high titer, high avidity anti-PspA antibodies. a. Anti-PspA IgG(H+L) antibody titer measured by ELISA at two, four, and seven weeks after vaccination. b. Antibody avidity indices measured seven weeks after vaccination. Error bars represent the mean ± the SEM.