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Pulmonary dry powder vaccine of pneumococcal antigen loaded nanoparticles

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Abstract

Pneumonia, caused by *Streptococcus pneumoniae*, mainly affects the immunocompromised, the very young and the old, and remains one of the leading causes of death. A steady rise in disease numbers from non-vaccine serotypes necessitates a new vaccine formulation that ideally has better antigen stability and integrity, does not require cold-chain and can be delivered non-invasively. In this study, a dry powder vaccine containing an important antigen of *S. pneumoniae*, pneumococcal surface protein A (PspA) that has shown cross-reactivity amongst serotypes to be delivered *via* the pulmonary route has been formulated. The formulation contains the antigen PspA adsorbed onto the surface of polymeric nanoparticles encapsulated in L-leucine microparticles that can be loaded into capsules and delivered *via* an inhaler. We have successfully synthesized particles of ~150 nm and achieved ~20 μg of PspA adsorption per mg of NPs. In addition, the spray-dried powders displayed a FPF of 74.31 ± 1.32% and MMAD of 1.70 ± 0.03 μm suggesting a broncho-alveolar lung deposition facilitating the uptake of the nanoparticles by dendritic cells. Also, the PspA released from the dry powders maintained antigen stability (SDS-PAGE), integrity (Circular dichroism) and activity (lactoferrin binding assay). Moreover, the released antigen also maintained its antigenicity as determined by ELISA.

Graphical abstract

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Abbreviations

CD, Circular dichroism;

CLSM, confocal laser scanning microscopy;

ELISA, Enzyme-linked immunosorbent assay;

L-leu, L-leucine;

NGI, next generation impactor;

NPs, Nanoparticles;

NCMPs, Nanocomposite microparticles;

PGA-co-PDL, poly(glycerol adipate-co-ω-pentadecalactone);

PspA4Pro, pneumococcal surface protein A;

PVA, polyvinyl alcohol;

SD, spray-drying;

SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Keywords

Nanoparticle;

Dry powder inhalation;

Pneumococcal surface protein A (PspA);

Nanocomposite microcarrier;

Dendritic cells;

Antigen delivery system

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1. Introduction

Pneumococcal diseases are infections caused by *Streptococcus pneumoniae*, also known as *pneumococcus*, which utilizes the respiratory tract as a portal of entry into the body. Pneumococcal diseases are classified into non-invasive pneumococcal diseases (otitis media, sinusitis, non-bacteremic pneumonia) or invasive pneumococcal diseases (IPD) (septicemia, meningitis, pneumonia) (Jambo et al., 2010). *S. pneumoniae* is the leading cause of bacterial pneumonia worldwide amongst the immunocompromised, the elderly, children under the age of 5, and adults in the developed world which is likely to increase with an aging population (Wardlaw et al., 2006).

Currently, two pneumococcal vaccine types are available, polysaccharide vaccine (23-valent) and conjugate vaccine (10 or 13-valent) (Bogaert et al., 2004 and Wang and Curtiss III, 2014). The conjugate vaccines have shown higher efficacy for invasive infections compared to polysaccharide vaccines, but have lower levels of protection against mucosal diseases, such as pneumonia. Furthermore, these vaccines do not protect against non-vaccine serotypes, which has led to the emergence of diseases caused by these serotypes (Hicks et al., 2007 and Singleton et al., 2007). Moreover, conjugate vaccines are expensive, complicated and time consuming to manufacture thus limiting their usage especially in the low and middle income countries (LMICs) where a significant burden of the disease is reported (Walker et al., 2013). Consequently, with more than 90 serotypes identified and a steady rise in diseases by non-vaccine serotypes being reported, further emphasis on identifying and developing alternate vaccine candidates to be employed in an effective vaccine delivery system has gained importance (Miyaji et al., 2013).

Pneumococcal infections are mostly preceded by colonization of the upper airways and nasal carriage is a primary source of infection in humans (Kadioglu et al., 2008). Therefore, an optimal vaccination strategy would be to deliver the antigen *via* a mucosal route providing protection against both the colonizing bacteria and invasive disease. In addition, the activation of the immune system requires the effective delivery of antigen to antigen presenting cells (APCs) such as dendritic cells (DCs) which process the internalized antigen thereby generating an immune response (Kwon et al., 2005).

Institutes such as US NIH and PATH have elaborated on the usage of pneumococcal proteins as alternate vaccine candidates for their ubiquitous presence across serotypes (Ginsburg et al., 2012). These proteins have the ability to protect against all serotypes thus preventing the prevalence of serotypes (Ginsburg et al., 2012 and Wang and Curtiss III, 2014). Among the different types of pneumococcal proteins such as pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), pneumolysin; PspA is one of the most promising candidates and has been widely investigated by several groups (Briles et al., 2000a and Briles et al., 2000bHaughney et al., 2013, Moreno et al., 2010, Ogunniyi et al., 2007, Ogunniyi et al., 2001 and Vadesilho et al., 2012). The main function of PspA is preventing the deposition of complement on the surface of the bacterium thereby inhibiting opsonization and phagocytosis (Ren et al., 2004a and Ren et al., 2004b). PspA was also shown to inhibit death by apolactoferrin at mucosal sites (Shaper et al., 2004).

Vaccination with PspA has shown to induce protective antibodies in humans (Briles et al., 2000b and Nabors et al., 2000). However, recombinant protein based vaccines can be poorly immunogenic generating low antibody responses in the absence of adjuvants or delivery

systems. Research is now focused on delivering antigens *via* particulate carriers where the antigen can be associated with the particles acting as delivery systems with the added benefit of augmenting the generated immune response upon uptake by APCs (O'Hagan, 2001). In addition, particulate antigens are known to generate a stronger immune response compared to soluble antigen (Koppolu and Zaharoff, 2013). As such, research has been focused on using polymeric nanoparticles (NPs) as delivery vehicles for antigen delivery (Kong et al., 2013 and Koppolu and Zaharoff, 2013). Lately, it has also been shown by different groups that polymeric particles have enhanced the immunogenicity of PspA (Anish et al., 2014, Haughney et al., 2013 and Kong et al., 2013).

A dry powder vaccine delivered *via* the inhalation route can overcome certain challenges such as invasiveness, cold-chain requirements, low stability and integrity of the antigen that are often associated with the traditional liquid based vaccines currently available in the market (Al-fagih et al., 2011, Blank et al., 2011 and Kunda et al., 2013). To address these issues in this study, a dry powder vaccine containing antigen adsorbed polymeric NPs for delivery *via* inhalation was successfully developed. Due to their low inertia, NPs are likely to be exhaled upon inhalation and thus have been encompassed in microparticles by spray-drying using L-leucine (L-leu) as a microcarrier (Kunda et al., 2015). Furthermore, the effect of the formulation process on the stability, integrity, antibody recognition and activity of PspA was investigated. In addition, NPs uptake by DCs was visualized.

2. Materials and methods

2.1. Materials

Acetonitrile (HPLC grade), glycerol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), human lactoferrin, nile red dye (NR), phosphate buffered saline (PBS) tablets, poly(vinyl alcohol) (PVA, MW 9-10 KDa; 80%), trifluoroacetic acid (TFA, HPLC grade), Tween 80® and antibiotic/antimycotic (100X) solution were obtained from Sigma–Aldrich, UK. L-leucine was purchased from BioUltra, Sigma, UK. Tissue culture flasks (25 and 75 cm^2) with vented cap, 96-well flat bottom and U shaped plates, acetone, dimethyl sulfoxide (DMSO) and tetrahydrofuran were purchased from Fisher Scientific, UK. Alpha minimum essential medium (α-MEM) and granulocyte macrophage colony-stimulating factor (GM-CSF) was purchased from Life technologies, UK. Fetal calf serum (FCS) heat inactivated was purchased from Biosera UK. Micro BCA™ protein assay kit was purchased from Thermo Scientific, UK. Dendritic cell lines, JAWS II (CRL-11904™) were purchased from American type culture collection (ATCC). The biodegradable polymer, poly(glycerol adipate-co-ω-pentadecalactone), PGA-co-PDL was synthesized as described previously (Thompson et al., 2006).

2.2. Preparation of nanoparticles and characterization

The polymeric NPs were prepared using the synthesized polymer as previously described (Kunda et al., 2015). Briefly, 200 mg of PGA-co-PDL was dissolved in 2 ml DCM and probe sonicated (20 μm amplitude) for 2 min under ice and upon addition to 5 ml of 10% w/v PVA formed an emulsion. This was then added drop wise to 20 ml of 0.75% w/v PVA under magnetic stirring at a speed of 500 RPM. The mixture was left stirring for 3 h at room

temperature to facilitate the evaporation of DCM. Nile Red, (NR) 0.5 mg was added to the organic phase to prepare dye loaded NPs for characterization by confocal microscopy.

Particle size, poly dispersity index (PDI) and zeta-potential were measured by laser diffraction using a laser particle size analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., UK). For NP suspensions, an aliquot of 100 μl was diluted with 5 ml of deionized water and for NP suspensions with protein adsorption, 2 mg of NPs were resuspended in 5 ml of deionized water, loaded into a cuvette and the measurements were recorded at 25 °C (n = 3).

2.3. Production and purification of PspA4Pro

The N-terminal α-helix and the first block of the proline rich region of PspA from clade 4 (PspA4Pro), fragment with cross reactivity with PspA from other clades (Moreno et al., 2010), was cloned into pET37b^{+} and expressed in *Escherichia coli* BL21 (DE3). The protein was produced and purified using previously published methods with slight modifications (Carvalho et al., 2012, Horta et al., 2012 and Horta et al., 2014). The production of recombinant PspA4Pro was performed in 5 l bioreactors using, fed-batch cultivation with defined medium containing glycerol as carbon source and lactose as inducer (Horta et al., 2012) or batch cultivation with complex medium containing glucose, glycerol and lactose for auto-induction (Horta et al., 2014). The purification method consisted of cell disruption in a continuous high pressure homogenizer, precipitation of the homogenate with cetyltrimethylammonium bromide, pellet removal by centrifugation, anion exchange chromatography in Q-Sepharose, cryoprecipitation and cation exchange chromatography in SP-Sepharose (Carvalho et al., 2012). The desired PspA4Pro purity (>95%) and the developed process also removed lipopolysaccharide, yielding acceptable levels of endotoxin (0.3–0.6 EU/ml) in the final product (Brito and Singh, 2011).

2.4. PspA4Pro adsorption and quantification

NP suspensions were collected by centrifugation (78,000 g, 40 min, 4 °C) and resuspended in 4 ml of PspA4Pro at a ratio of 100:20 (NP:PspA4Pro). The mixture was left rotating on a HulaMixer™ Sample Mixer (Life Technologies, Invitrogen, UK) for 1 h at room temperature and 20 RPM. The PspA4Pro adsorbed NPs were then centrifuged, as above and the supernatant analyzed for protein content using a micro BCA protein assay kit. The amount of protein adsorbed in μg per mg of NPs (n = 3) was calculated using Eq. (1):

$$\text{Adsorption } (\mu\text{gpermgofNPs}) = \frac{(\text{Initial proteinconcentration} - \text{Superna tan tproteinconcentration})}{\text{Amount of NPs}} \quad (1)$$

The particle size, PDI and zeta-potential of NPs with and without PspA4Pro adsorption were measured as above.

2.5. Preparation and characterization of nanocomposite microparticles

Nanocomposite microparticles (NCMPs) incorporating PspA4Pro adsorbed NPs were prepared by spray-drying using a Büchi B-290 mini spray-dryer (Büchi Labortechnik, Flawil, Switzerland) with a nozzle atomizer diameter of 0.7 mm and fitted with a high-performance cyclone. The NPs were suspended in L-leu solution at a NPs-to-carrier ratio of 1:1.5 w/w and spray-dried at a feed rate of 10% with an atomizing air flow of 400 L/h, aspirator capacity of 100% and an inlet temperature of 100 °C (outlet temperature of 45–47 °C). The dry particles were collected and stored in a desiccator at room temperature until further use. The resultant NCMPs were characterized as follows:

Yield: the dry powder yield% (w/w) was determined as the difference in the weight of the sample vial before and after product collection compared to the initial total dry mass (n = 3).

Morphology: spray-dried PspA4Pro adsorbed NPs/NCMPs were mounted on aluminum pin stubs (13 mm) layered with a sticky conductive carbon tab and coated with palladium (10–15 nm) using a sputter coater (EmiTech K 550X Gold Sputter Coater, 25 mA, 3 min), and visualized using scanning electron microscopy (FEI Quanta™ 200 ESEM, Holland).

2.6. *In vitro* release studies

The *in vitro* release studies were performed by adding 20 mg of PspA4Pro adsorbed NPs/NCMPs into Eppendorf's and then dispersed in 2 ml of PBS, pH 7.4. The samples were incubated at 37 °C and left rotating for 48 h at 20 RPM on a HulaMixer™ Sample Mixer (Life Technologies, Invitrogen, UK). At pre-determined time intervals up to 48 h, the samples were centrifuged (accuSpin Micro 17, Fisher Scientific, UK) at 17,000 g for 30 min and 1 ml of the supernatant removed and replaced with fresh medium. The supernatant was analyzed using the HPLC method as mentioned below. Each experiment was repeated in triplicate and the result was the mean value of three different samples (n = 3). The percentage cumulative protein released was calculated using Eq. (2):

$$\%\text{Cumulative protein released} = \frac{\text{Cumulative protein released}}{\text{Protein loaded}} \times 100 \quad (2)$$

2.6. High performance liquid chromatography method

A HPLC method was developed to quantify the amount of protein, PspA4Pro, present in a given sample. The optimum chromatographic conditions employed were as follows: HPLC system Agilent 1100 series (Santa Clara, CA, USA) equipped with a column (Aeris 3.6 μm C4 200A Wide Pore 4.6 mm i.d. ×150 mm length), security cartridge of the same material (Phenomenex, UK) and software for data processing, mobile phase was composed of (A) 0.1% w/v TFA in water and (B) 0.1% w/v TFA in acetonitrile with a gradient flow of A/B from 80:20 to 35:65 in 25 min, post-time 6 min, flow rate of 0.8 ml/min, injection volume of 100 μl, run temperature 40 °C, UV detection at 214 nm and pspA4Pro retention time of 14.9 min. The protein calibration curve was prepared from stock solution (1 mg/ml) in HPLC water and PBS (pH 7.4) to obtain the following concentrations: 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 200 μg/ml of protein (n = 9, R^2 = 0.999).

2.7. Investigation of PspA4Pro structure and activity

2.7.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The primary structure of PspA4Pro released from the NPs/NCMPs after spray-drying was determined by SDS-PAGE. A 9% stacking gel (Geneflow Limited, UK) containing 0.4% SDS and protein loading buffer blue (2X) was added to the samples in a 1:1 (v/v) buffer-to-sample ratio. Samples were loaded (25 μl per well) and the gel was run for 2.5 h at a voltage of 100 V with Tris-glycine-SDS PAGE buffer (10X). The gel was stained with colloidal coomassie blue and left overnight in distilled water for destaining and imaged using a gel scanner (GS-700 Imaging Densitometer, Bio-Rad) equipped with Quantity One software.

2.7.2. Circular dichroism (CD)

The secondary structure of the released PspA4Pro was determined by CD spectroscopy using a J-815 spectropolarimeter (Jasco, UK) at 20 °C as previously described (Kunda et al., 2015). A 10 mm path-length cell was used to perform five scans per sample at a scan speed of 50 nm/min over wavelength 260–180 nm at a data pitch of 0.5 nm and band width of 1 nm. The baseline acquired in the absence of samples was subtracted (Henzler Wildman et al., 2003) and the secondary structure of the samples was estimated using the CDSSTR method (reference protein dataset 3) from the DichroWeb server (Whitmore et al., 2010).

2.7.3. Antibody recognition test of PspA4Pro integrity

The protein integrity was determined by antibody recognition using Enzyme-linked immunosorbent assay (ELISA) as previously described by Haughney et al. (2013) with slight modifications. Briefly, released PspA4Pro (50 μl, 0.5 μg/ml) was coated on a high binding 96-well plate (Costar 96-Well Microplates, Cole-Parmer, UK) followed by incubation overnight at 4 °C. The PspA4Pro solution was removed from the wells and blocking buffer, PBS with 1% fish gelatin (Sigma–Aldrich, UK), was added and incubated for 2 h at room temperature. After incubation, the buffer was removed and the wells were washed with PBS containing 0.5% Tween 20 (PBS-T) (3×). The anti-PspA monoclonal antibody 22,003 (QED Bioscience, San Diego, CA) (100 μl, 1 μg/ml) was added to each well and incubated overnight at 4 °C. The plates were then washed with PBS-T (3×) followed by addition of alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Europe Ltd., UK) (100 μl, 0.1 μg/ml). The plates were incubated for 2 h at room temperature and 200 μl of alkaline phosphatase yellow (pNPP) liquid substrate buffer (Sigma–Aldrich, UK) was added and incubated for 15 min at room temperature to develop the ELISA. The colorimetric changes were measured at 405 nm using a microplate reader (Epoch, BioTek Instruments Ltd., UK). The ratio of absorbance between the released PspA4Pro and the native protein was represented as relative antibody recognition.

2.7.4. Lactoferrin binding

5 mg of PspA4Pro adsorbed NPs/NCMPs NP/NCMPs were incubated for 4 h in 500 μl PBS pH 7.4 at 37 °C with shaking. Samples were centrifuged at 15,700 g for 15 min, from which 30 μl of the supernatant was loaded onto a SDS-PAGE gel and samples were transferred to a nitrocellulose membrane, which was incubated with biotin-labeled lactoferrin (Human lactoferrin labeled with biotin using a Biotin Labeling Kit (Roche Life Sciences)). Lactoferrin binding was evaluated by incubation with streptavidin-HRP (BD Biosciences) and detection using Amersham ECL Prime Western Blot Detection Reagent (GE Healthcare).

2.8. Cell viability study

100 μl DCs were seeded (2.5×10^5 cells/ml) with complete medium (α-MEM containing ribonucleosides, deoxyribonucleosides, 4 mM L-glutamine, 1 mM sodium pyruvate supplemented with 20% FCS, 5 ng/ml murine GM-CSF and 1% antibiotic/antimycotic solution) in 96-well plates incubated at 37 °C, 5% CO_2 for 4 h. 100 μl empty NPs dispersions in complete medium were added at concentration (0–2.5 mg/ml) ($n = 3$), and 10% dimethyl sulfoxide (DMSO) as a positive control. The formulations were assayed for toxicity over 4 h incubation at 37 °C, followed by the addition of MTT (40 μl, 5 mg/ml) for 2 h. The complete medium was removed and replaced with DMSO (100 μl) in order to dissolve the formazan crystals. The absorbance was measured at 570 nm using a plate reader (Molecular Devices, SpectraMAX 190), and the percentage of viable cells was calculated as the absorbance ratio between NPs-treated and untreated control cells.

2.9. Cellular uptake of NPs by DCs

The uptake of NPs by DCs in cell culture medium and PBS were visualized using the confocal laser scanning microscopy (CLSM) (Carl Zeiss LSM 710, UK). The DCs (2×10^5 cells per well) were seeded onto an 8-well chambered #1 borosilicate coverglass system (Nunc Lab-Tek, Thermo Scientific, UK) and incubated at 37 °C for 48 h. NPs at concentrations of 2.5–5 μg/ml were incubated for 1 h at 37 °C with DCs. Following this, the supernatant was removed, washed three times with PBS and the DCs were fixed with 4% paraformaldehyde (Fisher Scientific, UK) for 10 min at room temperature. The DCs were then washed three times with PBS and 100 μl of 5 μg/ml wheat germ agglutinin antibody (WGA, Alexa Fluor® 488Conjugate, Life Technologies, UK) was added and incubated at 37 °C for 15 min. The excess antibody was removed by washing three times with PBS followed by staining the nucleus of DCs with 100 μl of 20 μg/ml 4′,6-diamidino-2-phenylindole, dilactate (DAPI, Sigma–Aldrich, UK), and then incubated for 10 min at room temperature. Excess DAPI was removed by washing three times with PBS.

2.10. *In vitro* aerosolization studies

PspA4Pro adsorbed NPs/NCMPs were weighed into capsules (Hypromellose, HPMC, capsule, size 3, each corresponding to 12.5 mg spray-dried powder and equivalent to 5 mg of NPs) and placed in a Cyclohaler® (Teva Pharmaceutical Industries Ltd.). A pump (Copley Scientific, Nottingham, UK) operated at a flow rate of 60 L/min for 4 s (corresponding to 4 KPa pressure drop across the inhaler) drew samples through the next generation impactor (NGI, Copley Scientific, Nottingham, UK). Samples were collected from the NGI using distilled water and left on a roller-shaker for 48 h for the PspA4Pro to be released from the NCMPs and then centrifuged using an ultracentrifuge. The supernatant was analyzed using HPLC method. The percentage deposition (%) of protein was calculated as the percentage ratio of protein amount collected in each stage to that of total amount of protein collected in all stages. The fine particle fraction (FPF, %) was determined as the fraction of emitted dose deposited in the NGI with $d_{ae} < 4.46$ μm, the mass median aerodynamic diameter (MMAD, μm) was calculated from log-probability analysis, and the fine particle dose (FPD, μg) was expressed as the mass of drug deposited in the NGI with $d_{ae} < 4.46$ μm ($n = 3$) in one actuation.

2.11. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's comparison using Minitab® 17 Statistical Software was employed for comparing the formulations, with statistical significant differences determined as $p < 0.05$. All values are expressed as mean ± standard deviation.

3. Results

3.1. PspA4Pro adsorption and quantification

The average adsorption of PspA4Pro onto NPs was 19.68 ± 2.74 μg per mg of NPs. Table 1 lists the particle size, PDI and zeta-potential of PGA-co-PDL NPs with and without PspA4Pro adsorption. The significant increase in particle size ($p < 0.05$) of NPs accompanied with a change in the surface charge can be ascribed to the adsorption of PspA4Pro onto NPs.

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Table 1. Particle size, PDI and zeta-potential of PGA-co-PDL nanoparticles (NPs) with and without pneumococcal surface protein A (PspA4Pro) adsorption (mean ± SD, *n* = 3).

	Without adsorption	With adsorption
Particle Size (nm)	203.90 ± 2.55[a,*]	322.83 ± 04.25[b,*]
PDI	0.205 ± 0.007	0.402 ± 0.018
Zeta-potential (mV)	−24.56 ± 0.50	−13.23 ± 0.11

[a]NPs characterised after centrifugation.

[b]NPs characterised after centrifugation.

*$p < 0.05$, ANOVA/Tukey's.

3.2. Characterization of nanocomposite microparticles

3.2.1. Yield, particle size and morphology

A reasonable yield of spray-dried NCMPs was obtained, with 55.55 ± 6.64% for the PspA4Pro adsorbed NPs/NCMPs. The SEM pictures (Fig. 1) of blank NPs/NCMPs revealed the shape to be irregular with a corrugated surface texture. The size of NCMPs calculated from SEM pictures was approximately 1.99 ± 0.25 μm.

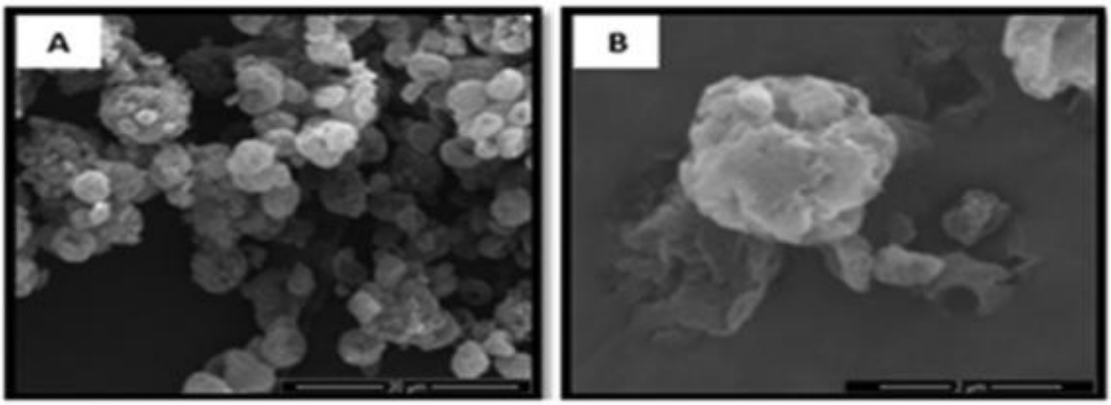

Fig. 1. SEM pictures of spraydried NPs/NCMPs. Scale bar (A) 20 μm and (B) 2 μm.

3.2.2. *In vitro* release studies

The cumulative percentage of PspA4Pro released over time from NPs/NCMPs (Fig. 2), indicated an initial burst release of 40.59 ± 4.94% was observed followed by continuous release of 89.81 ± 2.95% up to 5 h, eventually reaching 94.30 ± 2.90% over 48 h.

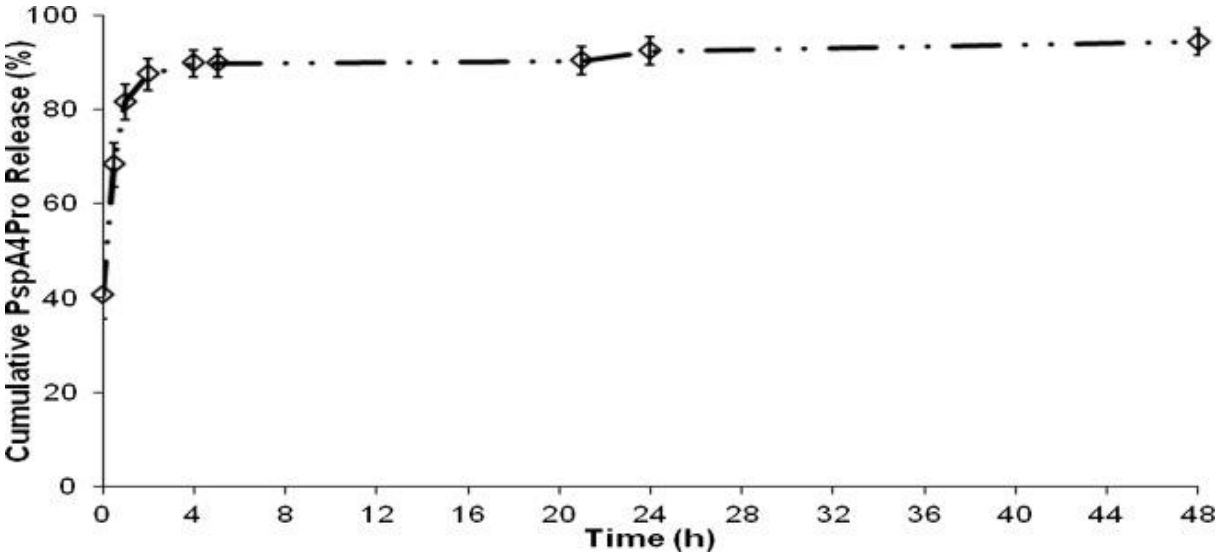

Fig. 2. *In vitro* release of pneumococcal surface protein A (PspA4Pro) from NPs/NCMPs in phosphate buffer saline, pH 7.4 (mean ± SD, $n = 3$).

3.2.3. *In vitro* aerosolization studies

The deposition data obtained displayed a FPD of 60.02 ± 8.56 μg (per capsule of 12.5 mg), FPF of 74.31 ± 1.32% and MMAD of 1.70 ± 0.03 μm suggesting that the formulation was capable of delivering efficient PspA4Pro to the bronchial-alveolar region of the lungs. Fig. 3 shows the percentage stage-wise deposition of PspA4Pro in NGI.

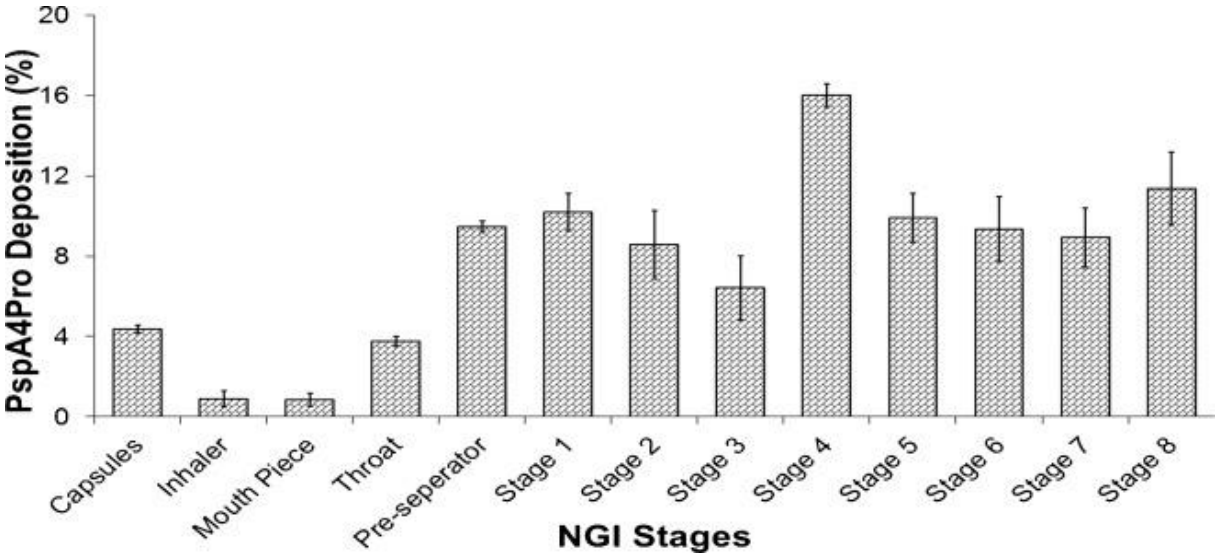

Fig. 3. The percentage deposition of pneumococcal surface protein A (PspA4Pro) stage-wise in next generation impactor (NGI) (mean ± SD, n = 3).

3.3. Investigation of PspA4Pro structure

The PspA4Pro released from the NPs/NCMPs was analyzed for primary and secondary structure using SDS-PAGE and CD, respectively. Fig. 4(a) indicates that the PspA4Pro released from NPs/NCMPs remained intact, based on the presence of a single band between 43 and 66 KDa, and no other bands of either small or large MW were visible indicating no degradation or aggregation, respectively.

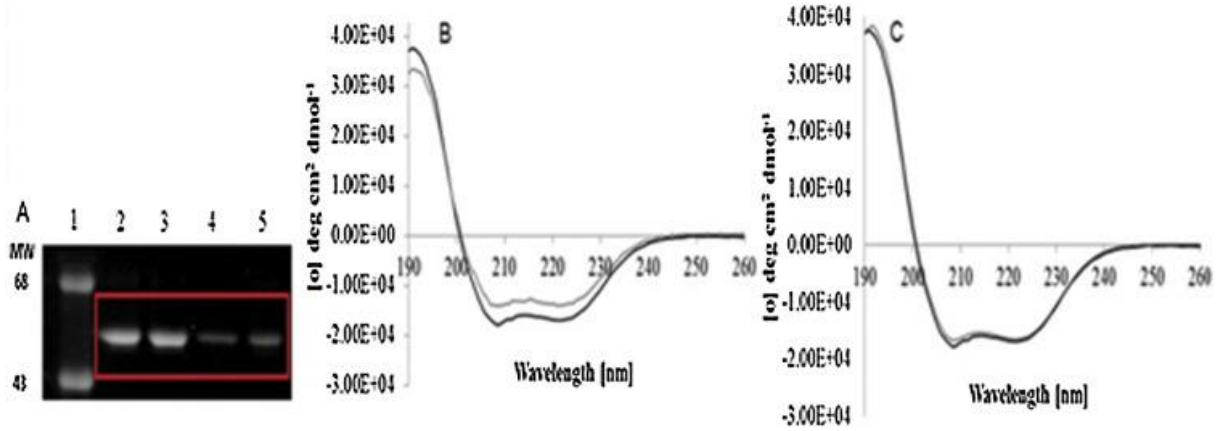

Fig. 4. (A) SDS-PAGE of Lane 1: MW standards, broad range (Bio-Rad Laboratories; Hercules CA, USA), Lane 2 & 3: pneumococcal surface protein A (PspA4Pro) standard, Lane 4 & 5: Released PspA4Pro from NPs/NCMPs after 48 h. Circular Dichroism (CD) spectra of pneumococcal surface protein A (PspA4Pro) standard (black) from: (B) adsorbed PGA-co-PDL NPs, supernatant (grey) and (C) PGA-co-PDL NPs/NCMPs, released (grey).

The conformational changes of PspA4Pro released from the NPs/NCMPs and PspA4Pro supernatant after adsorption onto NPs and centrifugation were investigated by CD spectroscopy. Fig. 4(b and c) represents the CD spectra of standard PspA4Pro, PspA4Pro supernatant, and PspA4Pro released from the NPs/NCMPs. The spectra indicate two characteristic minima at 208 and 222 nm, known to be associated with α-helical structure. The data for the spectra presented in Table 2 for standard PspA4Pro, PspA4Pro supernatant and PspA4Pro released, indicated that the predominant structure of the protein was helical displaying 64.5, 64 and 51% helicity, respectively. Hence, the α-helical content of PspA4Pro released when compared to the standard, decreased by 13.5%.

Table 2. The percentages of secondary structures of standard, supernatant and released pneumococcal surface protein A (PspA4Pro) samples.

Sample	Helix	Strand	Turns	Unordered
Standard PspA4Pro	64.50 ± 0.02	06.00 ± 0.00	07.50 ± 0.00	22.00 ± 0.01
PspA4Pro Supernatant	64.00 ± 0.02	07.00 ± 0.01	08.00 ± 0.01	20.50 ± 0.01
PspA4Pro Released	51.00 ± 0.03	21.00 ± 0.01	07.50 ± 0.01	20.00 ± 0.03

3.4. Antibody recognition test of PspA4Pro integrity and lactoferrin binding

The antibody recognition of released PspA4Pro from NPs/NCMPs was determined using ELISA with a specific anti-PspA monoclonal antibody and represented as relative antibody recognition. The assay measures the ability of released PspA4Pro to bind and be recognized by an anti-PspA antibody. Fig. 5(a) shows that the PspA4Pro released from the NCMPs maintained its integrity with relative antibody recognition of 0.97 ± 0.20 compared to PspA4Pro standard. To analyze the maintenance of important functional epitopes in the PspA4Pro released from NPs/NCMPs, the binding of PspA4Pro to lactoferrin was investigated. As shown in Fig. 5(b), PspA4Pro recovered from the NPs/NCMPs (lane 1) was able to bind to lactoferrin, indicating that the lactoferrin-binding site is intact in the sample. Lanes 2 and 3 show the binding of lactoferrin to control standard PspA4Pro.

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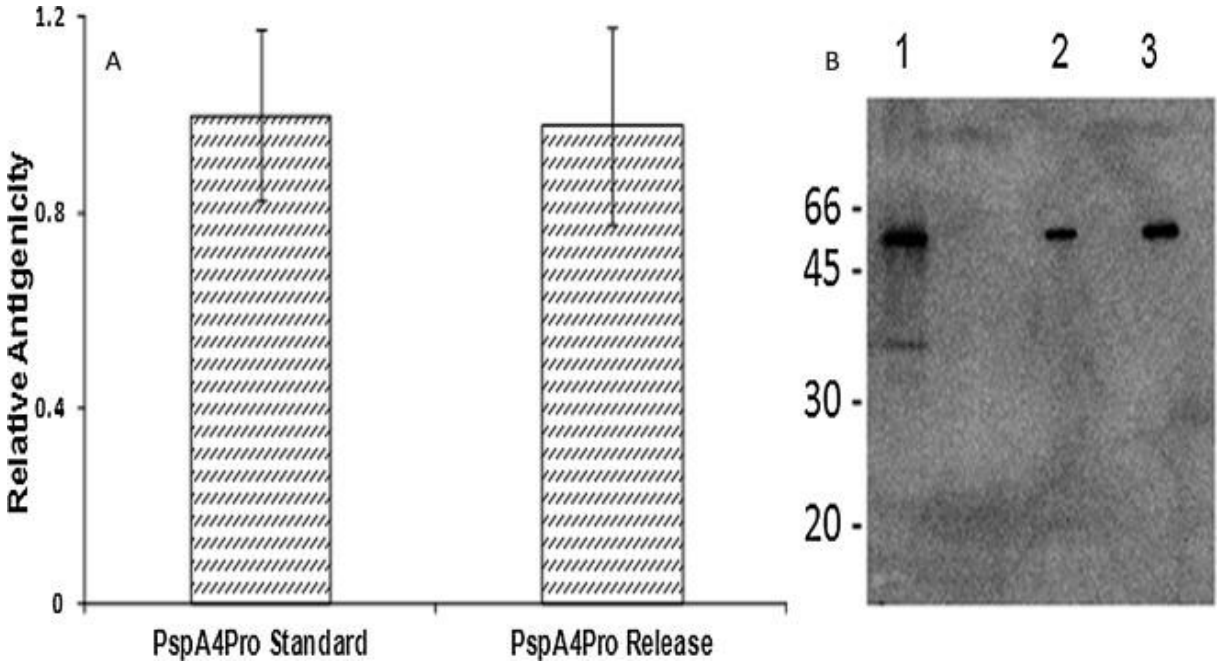

Fig. 5. (A) Antibody recognition of pneumococcal surface protein A (PspA4Pro) upon release from NPs/NCMPs, (B) Lactoferrin binding of released PspA4Pro from NPs/NCMPs. Lane 1: PspA4Pro released from NCMPs, Lane 2 & 3: PspA4Pro standard at 100 ng and 25 ng respectively.

3.5. Cell viability study & cellular uptake of NPs by DCs

The toxicity of non-loaded PGA-co-PDL NPs following 4 h incubation using a DC cell line, were assessed by the MTT assay. The NPs (Fig. 6) showed increased cell death with an increase in NPs concentration. The NPs showed up to 90% cell viability at 19.53 μg/ml concentration that reduced to ~55% at 1.25 mg/ml concentration. The uptake of NR/NPs by DCs was observed using confocal microscopy. The cell wall of the DCs (stained with WGA Alexa Fluor® 488), the nucleus (stained with DAPI) and the NPs (containing Nile Red) were observed under the green, blue and red channel, respectively. Fig. 7 shows the split view of DCs incubated with NR/NPs for 1 h in cell culture medium and PBS buffer, respectively. In addition, the orthogonal view NR/NPs uptake in cell culture medium and PBS buffer can be seen in Fig. 8.

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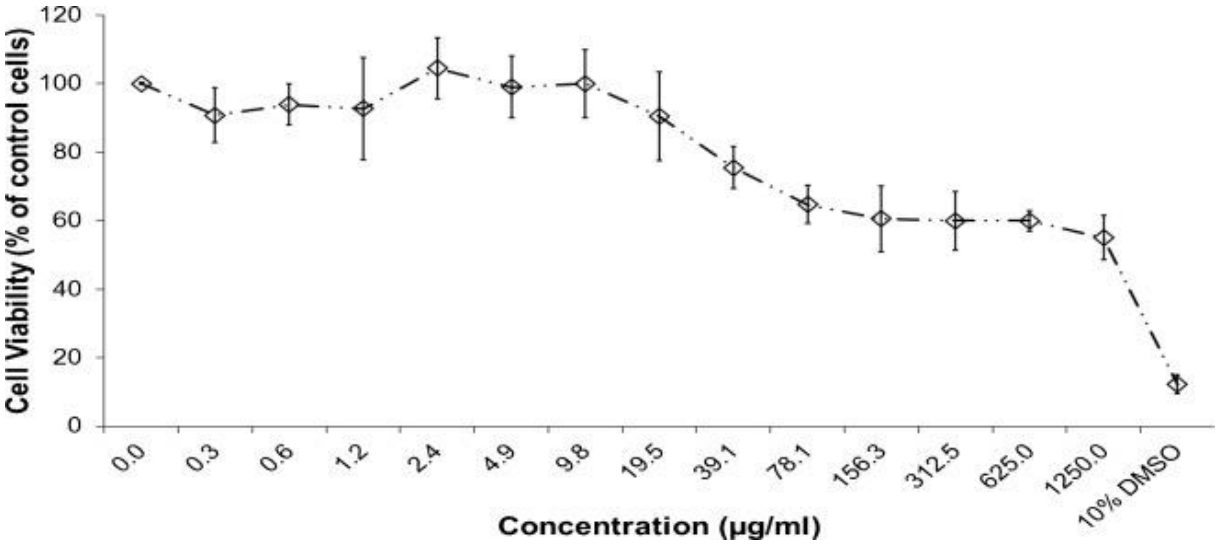

Fig. 6. Dendritic Cells (DCs) cell viability measured by MTT assay after 4 h exposure to PGA-co-PDL nanoparticles (NPs) (mean ± SD, n = 3).

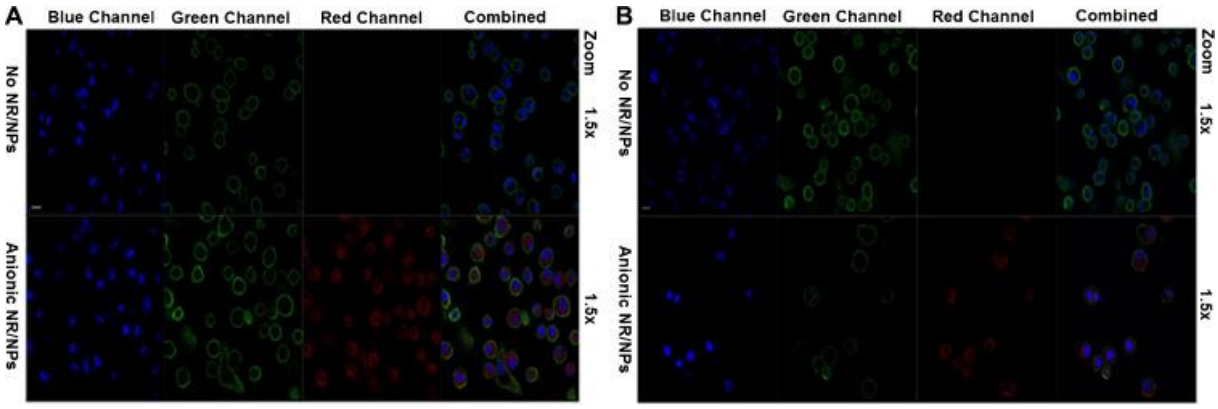

Fig. 7. Confocal microscopic images depicting a split view of dendritic cells (DCs) (40X lens) incubated in the absence and presence of Nile Red (NR)/nanoparticles (NPs) in (A) cell culture medium or (B) phosphate buffer saline (PBS) for 1 h (Scale bar—20 μm).

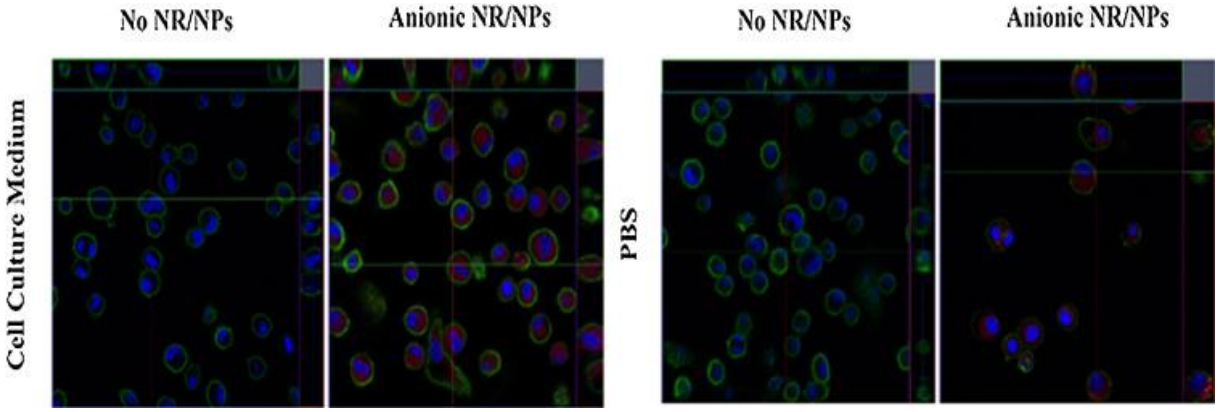

Fig. 8. Confocal microscopic images depicting an orthogonal view of DCs (40X lens) incubated in the absence and presence of Nile Red (NR)/nanoparticles (NPs) in cell culture medium and phosphate buffer saline (PBS) for 1 h.

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4. Discussion

Here, we utilized the optimum conditions from our previous study, of model protein adsorption onto polymeric particles (Kunda et al., 2015), for PspA4Pro adsorption onto PGA-co-PDL NPs. We spray-dried PspA4Pro adsorbed NPs producing NCMPs to develop a pneumococcal protein vaccine against S. pneumoniae to be delivered via dry powder inhalation. Additionally, we investigated the antigen stability and integrity in the final formulation.

Approximately 20 μg of PspA4Pro was adsorbed per mg of PGA-co-PDL NPs. The adsorption achieved with PspA4Pro was almost twice that of bovine serum albumin (BSA, 10 μg per mg of NPs) as reported by Kunda et al. (2015). This could be related to the MW of PspA4Pro (~43–50 KDa) being smaller than BSA (~66 KDa) thus providing more space on the NPs for PspA4Pro to adsorb. In addition, the elongated structure of PspA4Pro could enable the accommodation of more protein molecules on the surface of the NPs (Stolnik et al., 2001).

PspA has both negative and positive ends, however, in distilled water (pH ~ 7) the protein carries a net negative charge as the pH is greater than the pI of PspA (4.82). The adsorption process was performed in distilled water, and at this pH, the protein was negatively charged and hydrophobic interactions and hydrogen bonding would be the dominant forces of adsorption onto the NPs, which had a negative zeta potential. Adsorption of PspA4Pro onto the surface of the NPs led to an increase in the hydrodynamic diameter as evident from the increase in particle size. In addition, the decrease in surface charge could be due to the protein molecules orientation adopted on the surface of the NPs exposing only a small part of the protein. Similar results have been reported by Koppolu and Zaharoff (2013) and Gordon et al. (2008).

The PspA4Pro adsorbed NPs were spray-dried into NCMPs using l-leu as a microcarrier, displaying a rough and wrinkled surface texture. This has been widely reported in the literature, together with benefits for dry powder aerosolization (Seville et al., 2007, Sou et al., 2013, Tawfeek et al., 2011 and Tawfeek et al., 2013). The FPF (~74%) and MMAD (~1.7 μm) obtained for the PspA4Pro adsorbed NPs/NCMPs suggest an excellent aerosolization performance similar to that observed for BSA adsorbed NPs/NCMPs (FPF: ~77 % and MMAD: 1.21 μm) (Kunda et al., 2015).

The in vitro release data from PspA4Pro adsorbed NPs/NCMPs indicated a burst release of 40% with complete release (~94%) within 48 h. Though a high burst release was observed, this would not be a limiting factor for vaccination because any antigen released can still be taken up by DCs for processing and presentation, and that which is adsorbed onto NPs (intact) can also be taken up potentially giving an adjuvant effect as reported by Ghotbi et al. (2011), Cruz et al. (2012) and others (Carrillo-Conde et al., 2011, Cruz et al., 2012 and Ghotbi et al., 2011). Further, it has been established that for NPs to act as an immune potentiator or adjuvant, the attachment of NPs and antigen may not be mandatory; hence, the particles without antigen adsorbed can be taken up by DCs enhancing the immune response (Zhao et al., 2014). Moreover, studies in the literature have reported the uptake of particles by DCs within 1–2 h of incubation (Cruz et al., 2012, Karagouni et al., 2013 and Saleem et al., 2005).

The structure of PspA4Pro released from NPs/NCMPs was investigated using SDS-PAGE (primary structure) and CD (secondary structure). The SDS-PAGE of PspA4Pro released from the formulations confirmed that the protein had not degraded based on the presence of a single band. This shows that the protein remained intact during the adsorption, spray-drying process and desorption. In addition, the CD spectral data obtained for the released PspA4Pro confirmed the presence of α-helix and β-sheets. However, there is a decrease in the helical content observed (∼13%). This effect could be due to the adsorption and desorption process or spray-drying. However, the antigenicity of released PspA4Pro showed the ability to effectively recognize and bind to the anti-PspA monoclonal antibody. Furthermore, the lactoferrin-binding site of PspA4Pro is functional after release from the NPs/NCMPs. This suggests that despite the reduction of α-helix structure the functional part of the antigen is still active. The results obtained with SDS-PAGE, CD antibody recognition and lactoferrin binding provides an indication that PspA delivered via NPs/NCMPs is stable and retains antigenicity for generating an immune response.

PspA administered nasally (through a mucosal site) with an adjuvant into mice, a dose of only 100 ng was required to generate an immune response equivalent to that elicited by oral administration of 7.5 μg (Yamamoto et al., 1998). Considering that the NCMPs produced here are to be administered via inhalation i.e. through a mucosal site, the dose required would be ∼100 ng of PspA for mice, requiring NCMPs concentrations lower than 20 μg/ml. However, the dose of PspA4Pro would be different for human immunization via the pulmonary route (yet to be determined). In addition, it has been shown that the clinical dose required for pneumococcal polysaccharide vaccine administered via nebulized inhalation is estimated to be about 25 μg (Menzel et al., 2005 and Meyer et al., 2006). Furthermore, intramuscular administration of rPspA achieved antibody responses at 25 μg (Nabors et al., 2000). Although the mechanisms of immune response between polysaccharide and PspA are different; if we assume a similar dose is required for dry powder pulmonary delivery, then using PGA-co-PDL NPs/NCMPs we are able to deliver 60.02 ± 8.56 ug of PspA (FPD per capsule of 12.5 mg). Hence, we can adjust the amount of spray-dried powder to approximately 5.21 mg to achieve the relevant 25 μg dose.

It has now become evident that particulate antigens provide a Th1 type immune response (Couvreur and Vauthier, 2006, Gamvrellis et al., 2004 and Joshi et al., 2013). In addition, the size, charge, shape and site of delivery all affect the extent of DCs uptake and thus the immune response generated (Joshi et al., 2013), with NPs <500 nm showing higher uptake and activation of DCs and inducing stronger immune responses (Foged et al., 2005, Joshi et al., 2013 and Koppolu and Zaharoff, 2013). Here, to substantiate our claims of effective uptake by DCs upon producing NPs of size ∼300 nm, their uptake in JAWS II DC cell type was visualized using confocal microscopy. In cell culture media, NR fluorescence was detected when PGA-co-PDL NR/NPs were co-incubated with DCs for 1 h indicating the presence of NR/NPs within the DCs. However, Xu et al. (2009) have recently observed that NR, a lipophilic dye often used to stain intercellular lipid bodies, was released in the presence of cell culture medium and/or transferred directly to the cell membrane of contacting cells. It was also observed that the release of NR was significantly impeded in PBS. To investigate if the red fluorescence signals seen within the DCs is indeed the NR/NPs or the released NR, the uptake studies were repeated by co-incubating the NR/NPs with DCs in PBS medium. The presence of the red fluorescence was evident for PGA-co-PDL NR/NPs confirming their internalization by DCs thereby effectively endorsing the suitability of the size of NPs produced for vaccine delivery.

5. Conclusion

The results obtained above show that PspA4pro can effectively be adsorbed onto PGA-co-PDL NPs (~20 μg of PspA4Pro per mg of NPs). The stability (primary and secondary structure) of the released PspA4Pro was maintained and relative antigenicity has been retained. Moreover, the functional part of the antigen was active in the formulation as determined by lactoferrin binding. In addition, the aerosol properties (FPF of 74.31 ± 1.32% and MMAD of 1.70 ± 0.03 μm) indicate deposition in the respirable airways i.e. the bronchial-alveolar region of the lungs, ideal for antigen uptake by DCs. Moreover, PGA-co-PDL NPs when co-incubated with DCs showed internalization within 1 h. Further investigations would focus on determining the immunogenicity of the released PspA4Pro in vivo.

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