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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.

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; 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; Singleton et al., 2007). Moreover, conjugate vaccines are expensive, complicated and

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.

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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; 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,bHaughney et al., 2013; Moreno et al., 2010; Ogunniyi et al., 2007, 2001; 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,b). 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; 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; 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; 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; 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, 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).