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# Self-assembled particulate PsaA as vaccine against *Streptococcus pneumoniae* infection

## Abstract

*Streptococcus pneumoniae* is a human pathogen responsible for the majority of childhood pneumonia and media otitis cases worldwide. The diversity of its capsular polysaccharides (CPS) results in more than 91 serotypes of which at least 23 are virulent. Various CPS conjugated to immunogenic carrier proteins are currently licensed and provide protection against the infection caused by the respective serotypes but not against new and emerging virulent serotypes. In this study, we considered the conserved protein antigen PsaA, the pneumococcal surface adhesin A, in order to overcome the limitations of CPS antigens. The PsaA was translationally fused to a polyhydroxybutyrate (PHB) synthase which mediated production of PsaA displayed on PHB inclusions in recombinant *Escherichia coli*. This suggested that the PsaA fusion to the PHB synthase did not interfere with PHB synthase activity and its ability to mediate formation of nano-sized inclusions composed of a PHB core surrounded by the PHB synthase fused to PsaA. Isolated PHB beads showed a negative surface charge. Transmission electron microscopy analysis suggested that the PsaA fusion to the PHB synthase reduced the size of PHB beads from about 500 nm to 100 nm. The integrity and antigenicity of the fusion protein attached to isolated PHB beads was confirmed by SDS-PAGE, tryptic peptide fingerprinting analysis using MALDI-TOF-MS/MS and immunoblotting using a monoclonal anti-PsaA antibody. Mice immunized with PsaA displaying PHB beads produced high and specific IgG levels dominated by IgG1 isotype. While IgG1 titer were similar between soluble and insoluble PsaA, the IgG2 titers were strongly increased upon vaccination with insoluble PsaA i.e. PsaA displayed on PHB beads. Particulate PsaA-PHB beads elicited IgG antibodies recognizing PsaA in whole cell lysates of seven different serotypes of *S. pneumoniae*. This study suggested that PHB beads are suitable carriers for PsaA in order to induce a significant and specific Th-2-type immune response.

## Disciplines

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# Self-assembled particulate PsaA as vaccine against *Streptococcus pneumoniae* infection

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## Abstract

*Streptococcus pneumoniae* is a human pathogen responsible for the majority of childhood pneumonia and media otitis cases worldwide. The diversity of its capsular polysaccharides (CPS) results in more than 91 serotypes of which at least 23 are virulent. Various CPS conjugated to immunogenic carrier proteins are currently licensed and provide protection against the infection caused by the respective serotypes but not against new and emerging virulent serotypes. In this study, we considered the conserved protein antigen PsaA, the pneumococcal surface adhesin A, in order to overcome the limitations of CPS antigens. The PsaA was translationally fused to a polyhydroxybutyrate (PHB) synthase which mediated production of PsaA displayed on PHB inclusions in recombinant *Escherichia coli*. This suggested that the PsaA fusion to the PHB synthase did not interfere with PHB synthase activity and its ability to mediate formation of nano-sized inclusions composed of a PHB core surrounded by the PHB synthase fused to PsaA. Isolated PHB beads showed a negative surface charge. Transmission electron microscopy analysis suggested that the PsaA fusion to the PHB synthase reduced the size of PHB beads from about 500 nm to 100 nm. The integrity and

antigenicity of the fusion protein attached to isolated PHB beads was confirmed by SDS-PAGE, tryptic peptide fingerprinting analysis using MALDI-TOF-MS/MS and immunoblotting using a monoclonal anti-PsaA antibody. Mice immunized with PsaA displaying PHB beads produced high and specific IgG levels dominated by IgG1 isotype. While IgG1 titer were similar between soluble and insoluble PsaA, the IgG2 titers were strongly increased upon vaccination with insoluble PsaA i.e. PsaA displayed on PHB beads. Particulate PsaA-PHB beads elicited IgG antibodies recognizing PsaA in whole cell lysates of seven different serotypes of *S. pneumoniae*. This study suggested that PHB beads are suitable carriers for PsaA in order to induce a significant and specific Th-2-type immune response.

Keywords: Immunology, Vaccines, Biochemistry, Pharmaceutical science

## 1. Introduction

*Streptococcus pneumoniae* is considered as most important pathogen causing severe pneumonia, meningitis and middle ear inflammation [1]. The cell surface of *S. pneumoniae* contains various proteins and capsular polysaccharides (CPS) which play an important role in pathogenicity such as attachment and immune system evasion. The diversity of CPS contributes to more than 91 serotypes, while cell surface proteins were found to be more conserved and less variable [2]. Hence, *S. pneumoniae* cell surface proteins are increasingly considered as vaccine candidate antigens.

All licensed vaccines are conjugated CPS based and induce a specific serotype dependent protective immune response profile i.e. a strong and specific Th2-type response [3]. However, the emergence of new invasive serotypes not covered by existing vaccines demands attention.

Subunit vaccines based on conserved proteins/epitopes could induce serotype independent and more broadly protective immunity [3]. Due to their relevance for pathogenicity, cell surface proteins such as pneumococcal surface protein A (PspA), pneumococcal surface antigen A (PsaA) and pneumolysin (Ply) are currently being considered for vaccine development [4, 5, 6]. As subunit vaccines are often less immunogenic, adjuvant and/or immunogenic delivery systems are needed. Recently, polyhydroxybutyrate (PHB) beads (< 1  $\mu\text{m}$ ) displaying specific antigens had been demonstrated as effective antigen delivery system in the context of the intracellular pathogens [7, 8]. PHB is a polyester naturally produced by various bacteria [9]. Introducing PHB biosynthesis genes into heterologous expression hosts, allows the intracellular formation of discrete and spherical PHB inclusions [10]. This also resulted in PHB inclusions densely coated with proteins of interest [11, 12, 13, 14, 15, 16]. Translational fusion of proteins of interest to PHB synthase, PhaC, retained its PHB bead forming activity displaying the protein of interest at the PHB bead surface [13, 17]. PHB beads were bioengineered to

display antigens from intracellular pathogens like *Mycobacterium tuberculosis* and Hepatitis C virus. These particulate vaccine candidates elicited both Th1 and Th2 antigen specific immune responses resulting in protective immunity [8, 18, 19]. In this study it was conceived to engineer PHB beads displaying PsaA as possible antigen delivery system to develop a particulate vaccine against the extracellular pathogen *S. pneumoniae*.

## 2. Materials and methods

### 2.1. Bacterial strains, oligonucleotides, plasmids and cultivation condition

Bacterial strains, plasmids and primers used in this study are listed in Table 1. *E. coli* XL 1 blue was grown at 37 °C in Luria Bertani (LB) in presence of ampicillin (100 µg/mL). PHB beads and recombinant soluble protein was produced in recombinant *ClearColi* [20]. *ClearColi* was grown in LB Miller media supplemented with glucose 1% (w/v), ampicillin (100 µg/mL). Chloramphenicol (50 µg/mL) was only added to media used for PHB bead production.

### 2.2. Construction of plasmids mediating production of PHB beads displaying PsaA

The gene encoding PsaA (amino acids 22–309) was synthesized by Genscript Corporation (USA) employing codon optimization for *E. coli* [24]. This hybrid gene encoding a translational fusion of PsaA with PhaC was constructed and cloned into plasmid pET-14b as outlined in Fig. 1.

### 2.3. Construction of the plasmid encoding N-terminally His-tagged PsaA for production of soluble PsaA

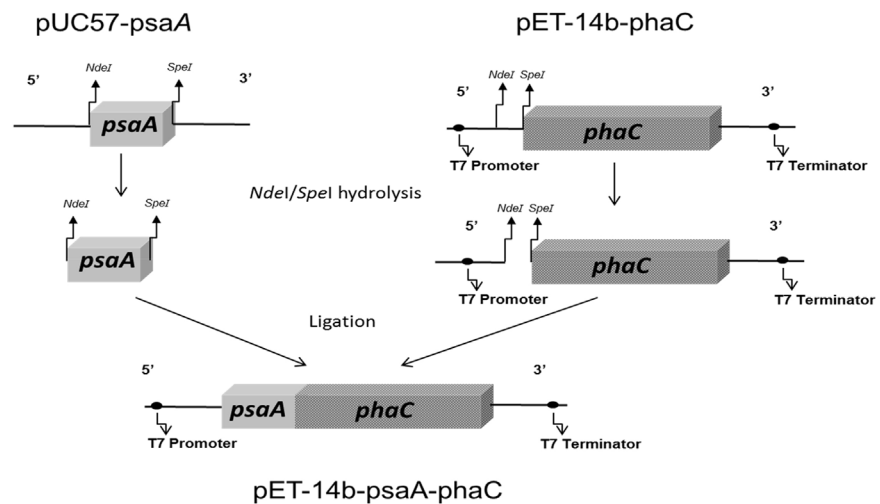
The gene encoding PsaA was amplified from pUC57-psaA by PCR using the corresponding primers psaA-fwr and psaA-rev. This introduced 6 histidine residues into the N terminus of PsaA in order to enable purification by immobilized Ni<sup>2+</sup> affinity chromatography. The gene encoding His6-PsaA was inserted into pET-14b\_NanA\_PhaC using hydrolysis with *NdeI* and *BamHI* to replace *nanaA*.

### 2.4. Production, isolation and purification of PHB beads

*ClearColi* harboring pMCS69 was transformed with pET-14b-psaA-phaC (encoding PsaA-PhaC fusion protein for production of PsaA displaying PHB beads) and pET14b-PhaC (PhaC wildtype control for production of PHB beads). Cells were cultivated and subjected to mechanical cell disruption. Beads were isolated and sterilized as previously described [25, 26].

**Table 1.** Description of bacterial strains, plasmids and oligonucleotides used in this study.

Strains, plasmid and primers	Characteristics	Review
<b>Strains</b>		
<i>XLI-Blue</i>	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI<sup>q</sup>ZΔM15 Tn10 (Tet<sup>r</sup>)]</i>	Stratagene
<i>ClearColi</i>	<i>F- ompT hsdSB (rB - mB -) gal dcm lon λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) msbA148 ΔgutQ ΔkdsD ΔlpxL ΔlpxM ΔpagP ΔlpxP ΔeptA</i> (LPS negative)	[18]
<b>Plasmids</b>		
pET-14b	<i>Ap<sup>R</sup></i> and T7 promoter	Novagen
pET-14b-phaC	pET-14b version, holding <i>phaC</i> gene fragment	[21]
pUC57-psaA	pUC57 version, ColE1 origin, <i>Ap<sup>R</sup></i> holding <i>NdeI/psaA</i> gene/ <i>SpeI</i> .	GenScript
pET-14b-psaA-phaC	pET-14b-phaC version, holding <i>psaA</i> gene fused to 3' end of <i>phaC</i> gene	This study
pMCS69	<i>Cm<sup>R</sup></i> ; T7 promoter, pBBR1MCS derivative containing <i>phaA</i> and <i>phaB</i> genes from <i>Ralstonia eutropha</i> co-downstream to lac promoter	[22]
pET14b_NanA_PhaC (reversed)	<i>Ap<sup>R</sup></i> and T7 promoter, containing <i>nanA</i> gene cloned to 3' end of <i>phaC</i> gene	[23]
pET14b- his6-psaA	<i>Ap<sup>R</sup></i> and T7 promoter, containing the <i>his6-psaA</i> gene inserted into the <i>NdeI/BamHI</i> sites of pET14b.	This study
<b>Primers</b>		
psaA fwd ( <i>NdeI</i> , underline restriction site)	5' AAACATATGCACCACCACCACCACCCTCGGGCAAAAAGGATACGACCTCGG3'	This study
psaA rev ( <i>BamHI</i> , underline restriction site)	5' AAAGGATCCTCATTTTCCAGACCTTCAGCGATTTTGTCCAG3'	This study



**Fig. 1.** Schematic presentation of the construction of plasmid pET-14b-psaA-phaC encoding the PsaA-PhaC fusion protein for formation of PHB beads in recombinant *Cleaveli*.

## 2.5. Production, isolation and purification of recombinant soluble protein

*Cleaveli* was transformed with pET-14b-his6-psaA (encoding His6-PsaA). Cells were cultivated and lysed for purification of His6-PsaA using the Ni-NTA Fast Start Kit (Qiagen, Germany).

## 2.6. Confirmation of the PhaC *in vivo* activity using transmission electron microscopy (TEM)

Cells harboring plasmid pET-14b-psaA-phaC and pET-14b-phaC, respectively, were analysed by TEM as described previously [20] to demonstrate the presence of PHB inclusions inside cells which is indicative of *in vivo* functionality of PhaC and its fusion protein variants.

## 2.7. Protein analysis

PsaA-PhaC and PhaC beads as well as soluble His6-PsaA were analysed by SDS-PAGE as previously described [27]. Immunoblot analysis was conducted as perviously described [28]. A monoclonal anti-PsaA antibody (Steroid & Immunobiochemistry Laboratory, Canterbury Health Laboratories, Christchurch, New Zealand) was used to identify the PsaA. All images were obtained using the GEL-DOC 2000 (Bio-Rad Laboratories, USA) and analysed using Image Lab Software (Version 3.0 build 11, Bio-Rad Laboratories, USA). Proteins were further identified by MALDI-TOF/MS. To confirm the PHB bead surface display and identity of PsaA, ELISA using goat anti-mouse IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) as secondary antibody as well as CLSM (confocal laser

scanning microscopy) using a fluorescently (Alexa fluor 488) labelled goat anti-mouse antibody (Sigma-Aldrich, St. Louis, MO) as secondary antibody were employed as previously described [29].

## 2.8. Measurement of the PHA bead size distribution and zeta potential

Size distribution of the particles and the zeta potential were measured using the Mastersizer 3000 particle sizer (Malven instrument, United Kingdom) and the Zetasizer Nano ZS (Malven instrument, United Kingdom), respectively. Samples were prepared as 0.1% (w/v) of the wet PHB beads in saline solution. The pH values were adjusted with HCl.

## 2.9. Analysis of immunological properties of PHB beads

### 2.9.1. Immunization schedule

Four groups were prepared containing each 6 animals between 5–6 weeks old (male, Balb/c mice). Mice were acquired from CENPALAB (Centro Nacional para la Producción de Animales de Laboratorio, La Habana, Cuba). Three doses of 4 µg of antigen plus 100 µg of alum salt (ALHYDROGEL, Brenntag Biosector, Denmark) were subcutaneously administered to each animal. Animals of the placebo group received only alum salt. Immunizations occurred at 0, 14 and 21 d. Blood samples were collected from the retro-orbital plexus at days 0, 14, 21 and 28 d after vaccination. Group 1 (G1) received 4 µg of PsaA displayed on PHBs, Group 2 (G2) received 4 µg of His6-PsaA antigen as pure soluble protein, Group 3 (G3) received 4 µg of PhaC wild type PHB beads. Group 4 received only alum salts.

### 2.9.2. Assessment of anti-PsaA antibody titers in mice

Anti-PsaA and anti-PhaC IgG levels were measured using an indirect ELISA. Maxisorp 96-well plates (NUNC) were coated using 0.5 µg/ml of His6-PsaA and incubation overnight at 4 °C. The plates were then washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T) and blocked for 30 min at 37 °C with PBS containing 1% (w/v) BSA. After three washes with PBS-T, plates were incubated with dilutions of serum samples from individual mice in PBS-T containing 1% (w/v) BSA and 12.6 mM of EDTA (PBS-TB), for 60 min at room temperature (RT). The initial dilution factor (DF) of all samples was 1600. After three washes with PBS-T, plates were incubated for 60 min with the secondary antibody, the goat anti-mouse IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) diluted 1:10,000 in PBS-TB. Plates were developed using the peroxidase substrate as described elsewhere [30]. A serum was considered as positive if the initial absorbance at a wavelength of 570 nm was equal or greater twofold of the pre-



immune serum value. Results were displayed as the reciprocal antibody titres, representing the DF required to obtain half of the maximum level of absorbance.

### **2.9.3. Analysis of the isotype IgG profile in sera**

The isotype IgG profile against PsaA was analysed by using an indirect ELISA as described above. Pools of sera from all animals at time 28 d from G1 and G2 were prepared and diluted 1:1600. To detect the various IgG isotypes, plates were incubated with the respective anti-IgG1, IgG2a, IgG2b and IgG3 antibodies (derived from goat) (Sigma-Aldrich, St. Louis, MO) using a DF of 1:2500. Plates were developed using the anti-goat IgG peroxidase conjugate and the respective substrate as described above. The results from duplicate experiments are represented as mean values plus standard deviation (Mean  $\pm$  STD).

### **2.9.4. Serotype coverage of induced anti-PsaA antibodies**

The specificity of sera from animals immunized with PsaA-PhaC PHB beads for PsaA in whole cell lysates of pathogenic *S. pneumoniae* serotypes 1, 3, 5, 6b, 7F, 14 and 23F was assessed by immunoblotting. Cell suspension were prepared in PBS (pH 7.0) and adjusted to an OD<sub>620</sub> 0.8–1. Then 300  $\mu$ l of these suspensions were mixed with denaturing buffer (DF 2). Samples were incubated at 95 °C for 20 min. Twenty  $\mu$ l of soluble sample were separated by SDS-PAGE. For immunoblotting pooled sera from all animals of G1 (DF: 2000) and G2 (DF: 500) were used to assess whether induced antibodies would detect PsaA in various *S. pneumoniae* serotypes.

## **2.10. Statistical analyses**

Graph Pad Prism 4.03 (San Diego, USA) software was used for statistical analysis of data. Results were reported as results of 6 animals and significant differences were calculated by the Kruskal–Wallis non-parametric test. When significant differences were found, the Dunn's post-test was used, considering significant differences when P was less than 0.05.

## **3. Results**

### **3.1. Construction of plasmids mediating production of the PsaA-PhaC fusion protein and His6-PsaA**

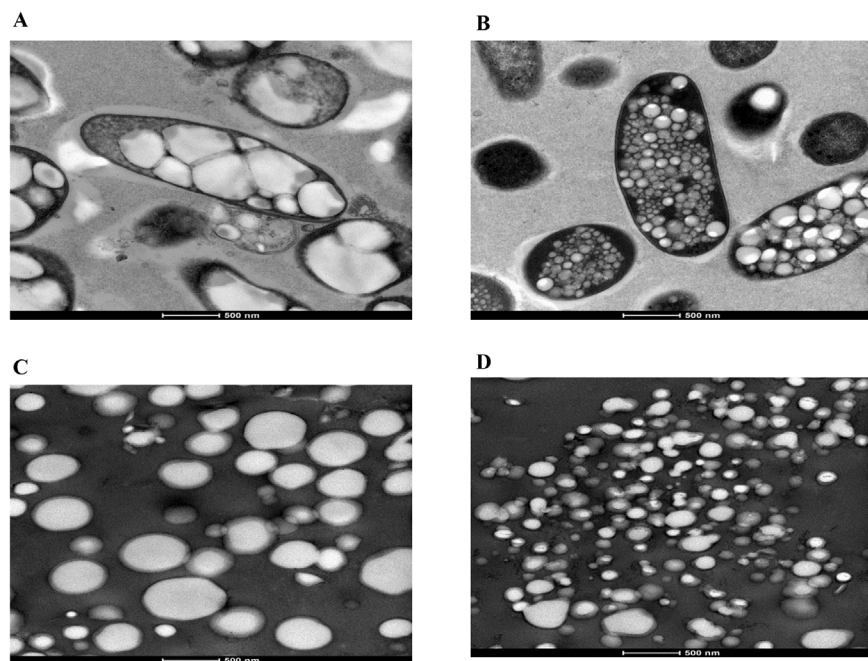
The strategy for construction of plasmids encoding PsaA from *S. pneumoniae* translationally fused to PhaC from *Ralstonia eutropha* for production of particulate PsaA is presented in Fig. 1.

In this study, the amino acid sequence of PsaA, the manganese ABC transporter, a manganese-binding adhesion lipoprotein, was selected from *S. pneumoniae*

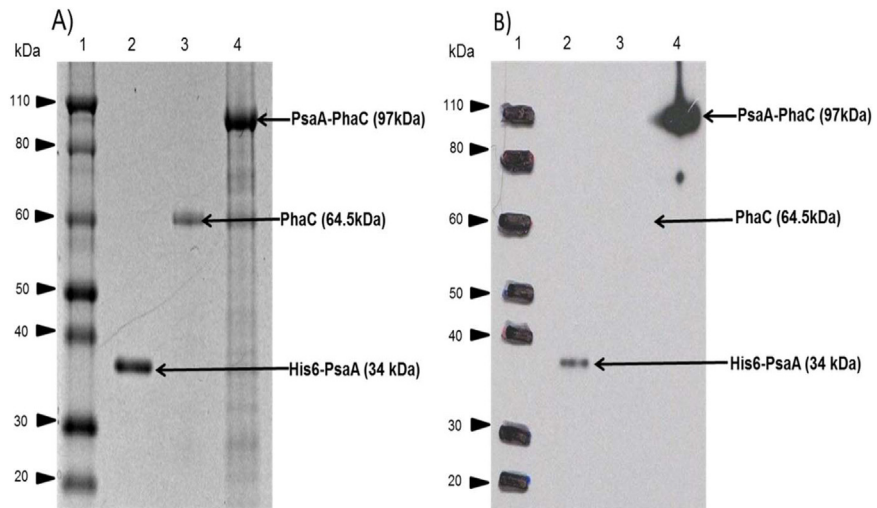
CGSP14 (GenBank number: ACB90875.1). This sequence has 100% identity with orthologous proteins found in strains R6 and TIGR4, and 98–99% identity with the other orthologues of various *S. pneumoniae* strains deposited in NCBI database. The gene encoding PsaA without its predicted signal sequence (1–21 amino acid residues) was synthesized as codon-optimized for *E. coli* (GenScript, USA). This synthetic gene was used to construct pET-14b-psaA-phaC. The recombinant soluble version of PsaA was produced as N-terminally His<sub>6</sub>-tagged protein and purified Ni<sup>2+</sup> affinity chromatography.

### 3.2. Production and characterization of PsaA displaying PHA beads

To produce PHB beads, *ClearColi* harbouring pMCS69 (Table 1) were transformed with either pET-14b-psaA-phaC or pET-14b-phaC (non-antigen displaying negative control). To confirm the formation of PHB inclusions indicative of PhaC functionality, cells and purified beads were observed by TEM (Fig. 2). Results showed that both plasmids mediated formation of distinct inclusions. However, PHB inclusions which formation was mediated by the PsaA-PhaC were smaller and more abundant when compared to inclusions formed by only PhaC (Fig. 2).



**Fig. 2.** TEM analysis of recombinant *Clearcoli* cells (pMCS69) harboring various plasmids and respective isolated PHB beads. A, cells harboring pET-14b-phaC (PhaC wildtype PHB beads); B, cells harboring pET-14b-psaA-phaC (PsaA-PhaC PHB beads); C, PHB beads isolated from cells harboring pET-14b-phaC; D, PHB beads isolated from cells harboring pET-14b-psaA-phaC.



**Fig. 3.** SDS-PAGE and immunoblot analysis of proteins attached to PHB beads and purified His6-PsaA. A, Coomassie blue stained SDS-PAGE gel; B, immunoblot of (A) developed using monoclonal anti-PsaA antibodies. Lane 1, Molecular weight (MW) standard (Novex® Sharp Pre-Stained Protein Standard, Invitrogen); lane 2, purified His6-PsaA (MW: 34 kDa) derived from cells harboring pET14b-his6-psaA; lane 3, PHB beads isolated from cells harboring pET-14b-phaC encoding PhaC (MW: 64.5 kDa); lane 4, PHB beads isolated from cells harboring pET-14b-psaA-phaC encoding PsaA-PhaC fusion protein (MW: 97 kDa). The arrows indicate the protein band of interest with the corresponding theoretical MW.

After isolation and purification, the protein profile of PHB beads and His6-PsaA was analysed by SDS-PAGE and immunoblotting (Fig. 3).

In Fig. 3A dominating protein bands are observed which correspond to the theoretical molecular weights of the various proteins of interest such as His6-PsaA (34 kDa) (soluble antigen control, PhaC (MW: 66 kDa) (mediating non-antigen displaying PHB bead formation) and PsaA-PhaC fusion protein (MW: 97 kDa) (mediating PsaA displaying PHB bead formation). The identity of proteins was further confirmed by immunoblotting using a specific anti-PsaA antibody (Fig. 3B) and by tryptic peptide fingerprinting analysis using Maldi-TOF/MS (Table 2).

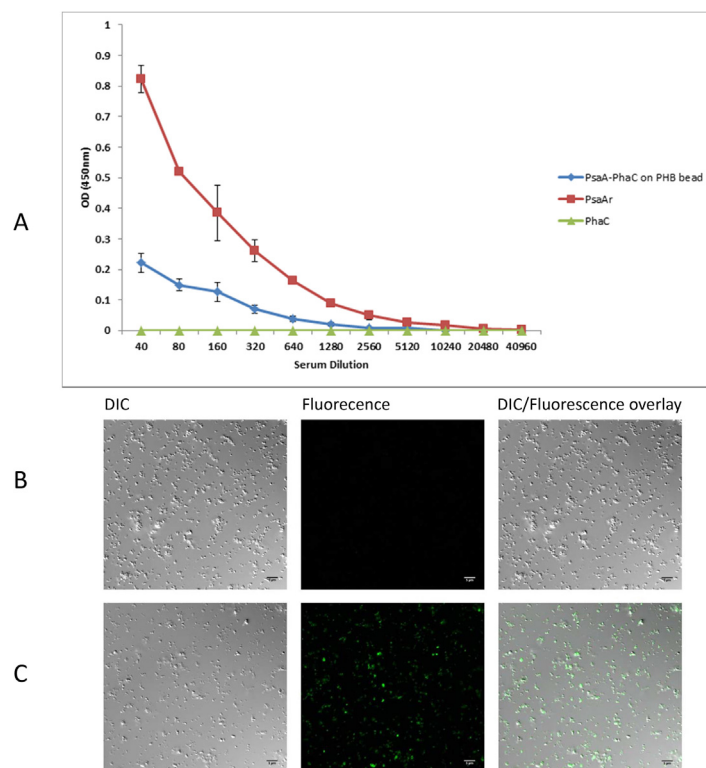
The SDS-PAGE analysis also showed a strong enrichment of the target proteins while the immunoblot confirmed identity and stability of the target protein with no obvious proteolytic truncation (Fig. 3). Densitometry suggested a purity of  $\geq 95\%$ .

**Table 2.** Tryptic peptide fingerprinting analysis (MALDI-TOF/MS).

Protein	Peptides fragment identified by MALDI-TOF/MS.
PsaA-PhaC fusion protein	N28-K58,G114-K132,V228-K243,V228-K243,T244-K267,V311-R326,I353-R364,D368-K380,A381-R391,A381-R391,R391-R398,F392-R398,T399-R404,F405-R415,F436-R459,F436-R459,L460-R470,I485-R497,N498-K524,Y540-R553,H554-R568,N569-R589,N569-R589,D596-R625,I603-R625,E661-R676,G677-K703,L746-R768,E769-K788,F791-K808,A854-R863,A866-R873,A866-R873.

In order to determine whether PsaA is displayed on the surface of the PHB beads, an ELISA and CLSM was employed which showed binding of the anti-PsaA antibody to the surface of PHB beads which formation was mediated by the PsaA-PhaC fusion protein (Fig. 4)

To determine the amount of PsaA- PhaC fusion protein attached to PHB beads, SDS-PAGE analysis including defined amount of BSA for generation of a densitometry based standard curve was applied. Densitometry using Image Analysis Software served to deduce the amount of protein per amount of PHB bead. The amount of PsaA-PhaC or PhaC per PHB bead is shown in Table 3. These data were used to calculate the amount of PHB beads to be injected per dose.



**Fig. 4.** Immunological assessment of PsaA display on the PHB bead surface. The specific monoclonal anti-PsaA antibody (see Materials and Methods) was used to detect PsaA at the surface of PHB beads by ELISA (A) and CLSM (B and C). A, Plates were coated with PsaA-PhaC beads, PhaC beads and soluble His<sub>6</sub>-PsaA. Anti-IgG HRP-conjugated secondary antibody was used to detect the bound primary anti-PsaA antibody. Data showed that the anti-PsaA antibody bound to PsaA on PHB beads as well as soluble PsaA while PhaC bead (negative control) did not show any binding. Each data point represented the mean of two replicates plus standard deviation. CLSM was further used to demonstrated PsaA surface display by detecting bound anti-PsaA antibodies with a secondary fluorescently labelled (Alexa fluor 488) antibody. PhaC beads (B) and PsaA-PhaC beads (C) were, after incubation with primary and secondary antibody, observed by CLSM. Fluorescent label of beads in the middle column (B, C) is indicative of PsaA display. DIC, differential interference contrast.

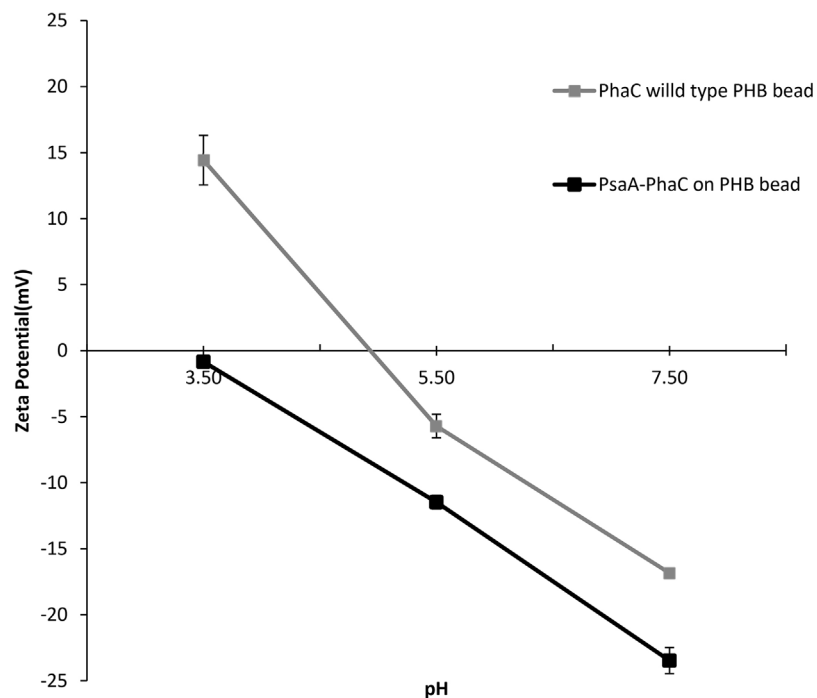
**Table 3.** PHB bead yield and composition.

Sample (Plasmids present in production strain)	Culture volume (L)	Wet bio-mass (g)	Wet PHA bead mass (g)	Antigen % in fusion protein	Antigen/wet Bead ( $\mu\text{g}/\text{mg}$ )
pET-14b-psaA-phaC	4	32.8	1.63	34	0.39
pET-14b-phaC	4	29.4	5.51	0	1

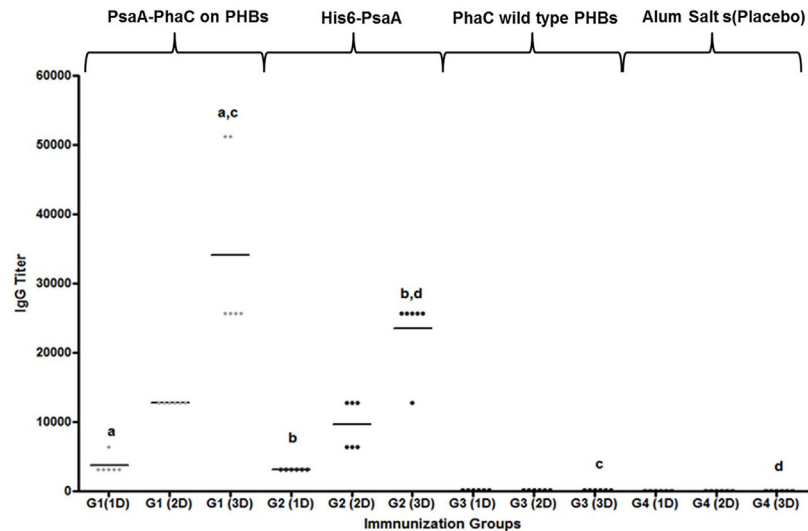
The Zeta potential of PHB beads was measured as a function of the pH (pH 3–7.5) suggesting a negative surface charge for both PHB beads (Fig. 5). However, display of PsaA further increased the negative surface charge.

### 3.3. Humoral immune response

During the immunization schedule all animals remained healthy and alive; they were gaining weight and showed no abnormal behaviour (data not shown). In the groups (G1 and G3) immunized with PsaA-PhaC and PhaC PHB beads small granulomas were detected (about 2 mm) at injection sites but no suppuration was observed. After euthanasia, the liver, lung, spleen and kidneys of the animals from groups G1, G2 and G3 showed no differences when compared with organs of animals which received the Placebo (G4).



**Fig. 5.** Correlation between zeta potential and pH of various PHB beads. Error bar represent the mean plus standard deviation between three measurements.

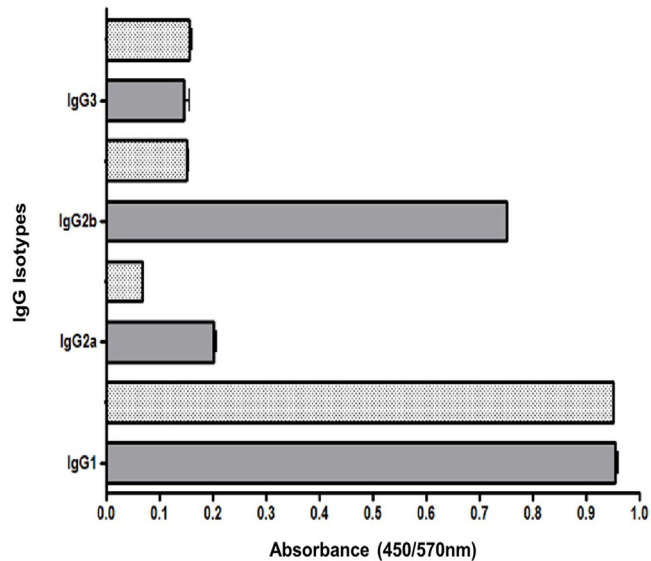


**Fig. 6.** Anti-PsaA IgG antibody response. Anti-PsaA IgG antibody titers were obtained by ELISA. G1 (grey diamond) group immunized with 4  $\mu$ g of PsaA fused to PhaC and displayed on PHA beads, G2 (black circle) group immunized with 4  $\mu$ g of His6-PsaA, G3 (black triangle) group immunized with 4  $\mu$ g of PhaC wild type bead (no antigen PHB bead negative control) and G4 (black square) (placebo) group immunized with only 100  $\mu$ g Alum salt. All of the immunogens were extracted from *E. coli* (*Clearcoli*, LPS free *E. coli* strain). Data are shown as reciprocal antibody titres, representing the dilution required to obtain half of the maximal OD value at 450/570 nm. <sup>a,b</sup>Statistical significant difference ( $p < 0.001$ ). <sup>c</sup>Significantly greater than the third dose of placebo and PhaC immunized control group ( $p < 0.01$ ), <sup>d</sup>Significantly greater than the third dose of placebo and PhaC immunized control group ( $p < 0.05$ ). No statistical differences were found after third dose between G1 and G2 ( $p = 0.240$ ).

IgG titers specific towards PsaA after each immunization dose are shown in Fig. 6. The absorbance value obtained in G1 and G2 showed induction of specific anti-PsaA antibodies when compared with control groups G3 and G4. Besides, the tendency of an increase in IgG titer after each dose was evident and significant for G1 and G2 (first dose vs third dose with ( $p < 0.001$ )). As expected the highest titers for these groups were achieved after the third dose presenting significant differences in comparison with G3 and G4 ( $p < 0.01$  and ( $p < 0.05$ )), respectively. No statistical significant differences were found between G1 and G2.

The humoral response was further studied, evaluating the IgG subclass profile in sera of animals 7 d after the last immunization. This analysis included subclasses IgG1, IgG2a, IgG2b and IgG3. IgG1 was the prevalent isotype elicited in G1 and G2. Interesting in the group immunized with PsaA-PhaC fusion protein on PHB beads IgG2b was the second prevalent subtype showing higher titers than the other isotypes (Fig. 7).

To assess whether induced anti-PsaA antibodies would recognize PsaA in whole cell lysates from different serotypes of *S. pneumoniae*, an immunoblot was performed using pooled sera from G1 and G2 obtained 7 d after the last



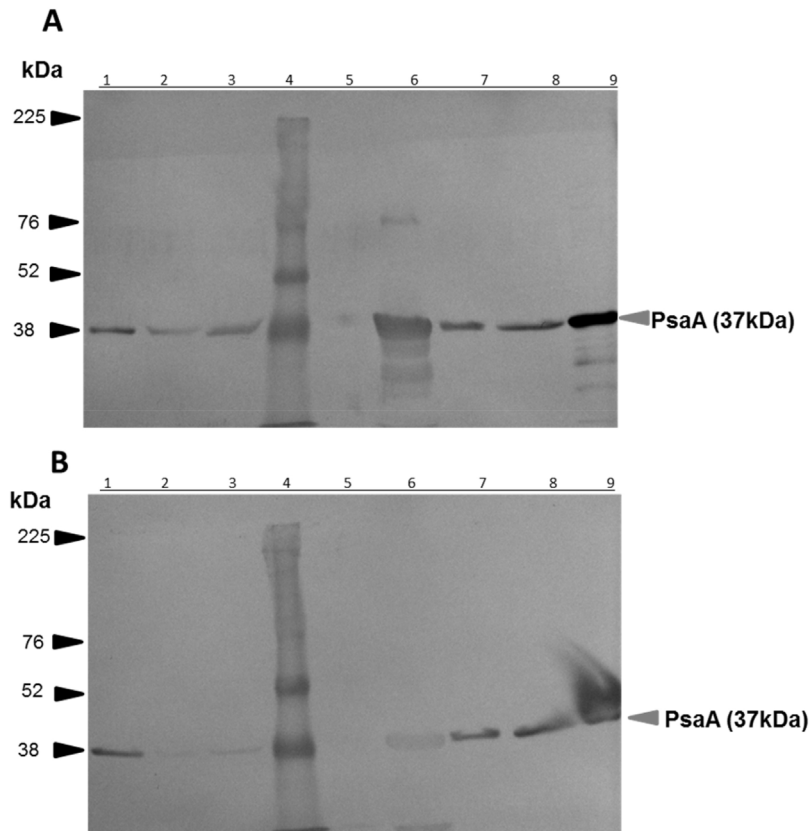
**Fig. 7.** Isotype IgG profile evaluated by direct ELISA using ELISA plates coated with 0.5  $\mu\text{g}$  of soluble His6-PsaA. The results are expressed as the average plus STD of absorbance readings at 450/570 nm using pooled of sera (6 animals) from G1 and G2. The sera were collected 1 week after third dose. The STD bars are only indicated in plus direction. G1 (grey bars) group immunized with 4  $\mu\text{g}$  of PsaA displayed on PHB beads, G2 (black point bars) group immunized with 4  $\mu\text{g}$  of soluble His6-PsaA. IgG1 is the prevalent isotype in both groups.

immunization (Fig. 8). Antibodies in sera of both groups G1 and G2 were predominantly detecting a protein with an apparent molecular weight of 37 kDa which corresponds with the theoretical molecular weight of PsaA and aligned with the purified PsaA. Interestingly, to get a visible band the sera from G2 had to be 4-fold more concentrated than from G1 suggesting enhanced production of the antibodies elicited after immunization with PsaA displayed on PHB beads versus its soluble counterpart.

#### 4. Discussion

Current vaccines against *S. pneumoniae* infections are limited to protect against certain serotypes excluding important invasive pathogenic serotypes. Hence a serotype independent broadly protective vaccine, such as a vaccine based on conserved cell surface proteins could provide an alternative approach toward vaccine development.

PsaA has been considered as one of the most important vaccine candidate antigens as it plays a critical role during pathogenesis and is conserved among virulent strains. It induces both B-cell and T-cell responses. In this study we evaluated the concept of improving delivery and immunogenicity of PsaA by its display on PHB inclusions. Antigen displaying PHB beads (<1  $\mu\text{m}$ ) had been produced by engineered *E. coli* and induced both Th1 and Th2 immune responses as well as



**Fig. 8.** Recognition of PsaA in various serotypes of *S. pneumoniae* by sera from mice immunized with PsaA displayed on PHB beads or soluble PsaA. A, immunoblots using sera (diluted 1:2000) from mice immunized with PHB beads displaying PsaA (G1). The corresponding SDS-PAGE of whole cell lysates of various *S. pneumoniae* serotypes is provided in Supplemental material (Fig. S1). B, immunoblot using sera (diluted 1:500) from mice immunized with soluble PsaA (G2). Lanes, 1, 2, 3, 5, 6, 7 and 8 correspond to cell lysates from *S. pneumoniae* serotypes: 1, 3, 5, 6b, 7F, 14, 23F, respectively; lane 4, MW standard and lane 9 soluble purified PsaA. The grey arrows indicate the protein corresponding to the molecular weight expected for PsaA.

mediated protective immunity against tuberculosis and hepatitis C [8, 18, 31]. Antigen PHB beads are presumable taken up by phagocytosis of antigen presenting cells (APCs) which creates an antigen depot and allows cross presentation on MHC class I and class II, which would further enhance PsaA immunogenicity [8, 29, 31].

In addition, it was shown that the co-administration of Th1-promoting adjuvants to protein-based *S. pneumoniae* vaccine candidates induced protective immunity [32]. This suggested that the Th1 immune response might contribute to protective immunity against the extracellular pathogen *S. pneumoniae*. This did further justify considering particulate vaccine such as PHB beads, known to induce Th1 responses, for formulation of a *S. pneumoniae* vaccine.

To produce PsaA displaying PHB beads, a hybrid gene was constructed (Fig. 1) which encoded a single chain fusion protein of PsaA and the PHB forming enzyme,



PhaC. The fusion protein mediated production of PHB inclusions in the *ClearColi*, an endotoxin-free mutant of *E. coli* [33] (Fig. 2). Interestingly, the fusion of PsaA to PhaC resulted in formation of smaller PHB beads (<200 nm) than observed with only PhaC suggesting an impact of PsaA on self-assembly of PHB beads inside the cell (Fig. 2). Protein and immunoblot analysis confirmed that PHB beads displayed the full-length PsaA at high-copy number with only minor indication of degradation (Figs. 3 and 4, Table 2). PHB beads as antigen carrier isolated from recombinant *E. coli* were recently demonstrated to induce only antigen specific responses, while potentially co-purifying *E. coli* proteins did not induce a detectable responses [19]. As the surface charge of particulate vaccine might impact cellular uptake and antigen processing, we assessed the Zeta potential of isolated PHB beads which resulted in a negative charge at physiological pH with an increased negative Zeta potential for the PsaA-PHB beads (Fig. 5). Particulate vaccine formulations which are designed for improved uptake of antigens by APCs are known to activate the NLRP3 inflammasome, which enhances efficacy of the vaccine [34]. Activation of the NLRP3 inflammasome was found to dependent on the surface charge of the vaccine particle.

To compare immunological properties of soluble and particulate PsaA, the soluble PsaA was produced in *ClearColi* and purified by affinity chromatography to  $\geq 95\%$  purity (Fig. 3). Vaccination experiments showed that IgG levels elicited by PsaA on PHB beads were significantly greater than those induced by PHB beads and within the placebo group ( $p < 0.01$  and  $p < 0.05$ , respectively). Antibodies against bacterial antigens like cell surface proteins (e.g. PsaA or PspA) were shown to interfere with binding and internalization by the mucosal-nasopharyngeal cells, impairing entrance of the pathogen into blood circulation [35, 36]. In particular, the results obtained by De et al. [37] after immunization with PsaA, reinforces the above stated. *In vitro* studies further demonstrated that anti-PsaA antibodies inhibited adherence of various *S. pneumoniae* serotypes to nasopharyngeal human carcinoma cells [38]. PsaA trapped in alginate microspheres was demonstrated to mediate reduction of *S. pneumoniae* colonization resulting in protection against pneumonia and septicaemia [39].

Production of human IgG1 was related to clearance of extracellular pathogens by activating phagocytosis of pathogens by macrophages (and other phagocytic cells) through Fc receptor antibody interaction. Here immunization with PsaA displaying PHB beads induced predominantly IgG1 (Figs. 6 and 7). This result agrees with the study presented by Palmiappan et al., [40] where oral immunization with a *S. pneumoniae* strain EF3030 promoted significant PsaA-specific titers of subclasses IgG1 and IgG2a [40]. However, PsaA displayed on PHB beads induced IgG2b as the second predominant IgG subclass (Fig. 7). These subclasses (IgG2a/b) of antibodies are generally identified as part of a Th1 immune response usually associated with an immune response to intracellular pathogens. However, studies

on a murine pneumococcal carrier model illustrated the induction of IgG2b and IgG3 to be important against *S. pneumoniae* strain P1121 infection [41].

Due to their more conserved nature protein-based vaccines are potentially more broadly protective against various serotypes of *S. pneumoniae* as they induce a serotype independent immune response. To assess whether PsaA on PHB beads induced production of cross-reactive antibodies, we tested reactivity of whole cell proteins of serotypes 1, 3, 5, 6b, 7F, 14, 23F with antibodies in pooled sera of vaccinated mice (Fig. 8). These data clearly suggested that serotype independent anti-PsaA antibodies were induced by PsaA displaying PHB beads and by soluble PsaA. However, anti-PsaA antibody titers in response to PsaA displaying PHB were greater than antibody titers induced by soluble PsaA. Our results agree with previous studies where PsaA was suggested to be conserved among serotypes suitable for use as antigen for broadly protective vaccines [42, 43, 44]. The PsaA displayed in the surface of PHB beads was selected from the strain CGSP14 which PsaA is identical to PsaA of strains R6 and TIGR4 (Serotype 14) and shows 98–99% amino acid sequence identity with all other deposited PsaA protein sequences in the current NCBI database [24, 45]. Overall, this study demonstrated that bioengineering can be used to produce immunogenic PsaA displaying PHB beads, which mediate a serotype independent PsaA specific antibody response. Hence the PHB bead based particulate vaccine approach holds the promise to be applicable not only for protection against intracellular pathogens but also extracellular pathogens such as *S. pneumoniae*.

## Declarations

### Author contribution statement

Majela González-Miro: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Laura Rodríguez-Noda, Mildrey Fariñas-Medina: Performed the experiments.

Dagmar García-Rivera: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Vicente Vérez-Bencomo: Contributed reagents, materials, analysis tools or data.

Bernd H.A. Rehm: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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## Competing interest statement

The authors declare the following conflict of interests: Bernd H.A. Rehm is founding inventor, shareholder and CTO of PolyBatics Ltd which commercialises the PHA bead technology.

## Additional information

No additional information is available for this paper.

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