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Research Article

Process Development for Obtaining a Recombinant Protein Pneumococcal Intranasal Vaccine Candidate

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Abstract

Pneumonia represents considerable infection rates in young children. In 2015, this disease was responsible for 15% of the total deaths in children worldwide. The vaccines available contain 10 to 23 of the more than 90 pathogen's serotypes, and it's obtained through fermentations and purifications of the capsular polysaccharides of each serotype. This work proposes a process optimization of only one recombinant protein antigen, the PsaA (pneumococcal surface adhesion A), potentially capable to act against most of all prevalent serotypes of *Streptococcus pneumoniae* and suggests a formulation for intranasal administration. Based on protein expression in prokaryotic system and purification, this process reached high purity levels and satisfactory yield. Studies of expression level verified that fermentation time could be initially reduced from 16 to 10 hours. Purification steps showed good resolution using anion exchange chromatography (DEAE Sepharose™ FF). For desalting, comparative studies observed that gel filtration technique should be replaced for tangential cross flow filtration, which is better to scale-up using 10 kDa membranes. The developed process resulted in overall yield of 29%, based on BCA analysis. SDS-PAGE analysis after purification showed a PsaA band corresponding to 99% of identified proteins. It was also efficient in DNA and endotoxin clearance. As formulation proposal, chitosan was used to prepare the antigen capsules, using different polymer concentration, which can be considered as an intranasal delivery system. This data show that this process could be promising to obtain recombinant vaccines.

Keywords: Pneumococcal vaccine; PsaA; Protein vaccine; Chitosan

Introduction

Pneumonia infection usually affects respiratory tract and lungs, and it is the largest infectious cause of death, being responsible for 920,136 deaths in 2015 for young children (under five years old) worldwide, representing 15% of total fatalities in this age. The main infectious agent of pneumonia, the bacterium *Streptococcus pneumoniae*, is normally found at nose or throat and can spread by droplets from a cough or sneeze [1]. *S. pneumoniae* is capable to cause serious severe invasive disease, but it can colonize individuals who do not have symptoms, suggesting that colonization represents the primary selective force forth is bacterium, [2] but as transmission occurs mainly by respiratory tract including from asymptomatic individuals, mucosal immunization mechanisms must be constantly evaluated.

The antiphagocytic capsule, the main virulence factor of *Streptococcus pneumoniae*, is the base for bacterial classification, and until now, 94 different serotypes were identified [3]. Added to this, about 20 within these serotypes are responsible for 80% of all infections by *S. pneumoniae* in the world. Therefore, this diversity represents a serious obstacle to develop a universal vaccine [4,5]. The first vaccines against *S. pneumoniae* were available in 1911, with clinical trials using whole cells vaccine. Since then, the importance of capsular polysaccharides as antigenic, and the need to develop an efficient and polyvalent capsular vaccine were recognized. Thus, in 1977 the 14-valent and in 1983 the 23-valent vaccines were developed, but was not effective in young children [6]. Vaccines chemically coupled to a carrier protein where then presented as solution to generate immunologic memory in children and the first Pneumococcal Conjugated Vaccine (PVC) was a heptavalent in 2000. The 10-valent, 13-valent and 15-valent PVC's are also available in the market [6,7]. In Brazil the National Immunization Program adopted the 10-valent vaccine and it is supplied by Bio-Manguinhos, at Oswaldo Cruz Foundation. However, as the other PVC's, the 10-valent conjugated vaccine demands high-cost process production, because is comprised by fermentation of each bacterial serotype (to obtain specifics polysaccharides), purification, chemical coupling, purification of each conjugated, and finally formulation and final processing.

In the last decades, as potential vaccine antigen candidates, virulence proteins have been investigated as alternative to expensive conjugated vaccines [8]. Proteins have several advantages, because are T-dependent antigens capable to produce immunologic memory in children; these proteins can be engineered to obtain higher levels of expression with lower costs in production, resulting in more affordable vaccines. Some studies shown that conjugated vaccines had reported fails in otitis media protection, however, some proteins can better help against this kind of infection and a protein antigen could be used as carrier in polysaccharide vaccines to elevate protection against otitis media [9], reinforcing the use of proteins as vaccines.

Among the protein candidates, the PsaA (Pneumococcal surface adhesin A), a 37-kDa cell membrane-associated and surface-exposed lipoprotein, is composed of 309 amino acids [10]. It belongs to the group of mettalo-lipoproteins and its function is to transport Mn^{+2} and Zn^{+2} to the cytoplasm of the bacterium [5,11]. The PsaA protein is part of the primary key acquisition of Mn^{+2}, and mediates the acquisition of this essential element from the extracellular environment, therefore, the PsaA protein plays a vital role in virulence of *S. Pneumonia* [11]. Due to its high degree of conservation among serotypes –showing an identity of 97–100% among the *S. pneumoniae* genomes recorded in the NCBI database, and its capacity to induce immunity against *S. pneumonia*, PsaA can be considered as an important candidate for a vaccine formulation. Because it's able to cover a broad range of pneumococcal infections and induce protection against nasopharyngeal colonization, which is the most important form of acquiring pneumococcal infection [8,12].

In this document, an optimization of the PsaA protein obtaining process is presented. The protein is considered as an antigen candidate

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for an intranasal vaccine formulation, which is obtained from the expression of a recombinant microorganism (not from a classical fermentation of the pathogen), in an affordable alternative route to conjugated vaccines.

As formulation proposal chitosan was presented using precipitation-coacervation method to be applied for intranasal administration.

Intranasal route has been recognized to bring benefits as the direct transport of drugs to be absorbed into the systemic circulation [13]. Whereas the presence of specific antibodies in mucosal surfaces was reported to be the first barrier against pathogens entrance, the most effective way to induce mucosal immunity is to apply the vaccine directly to the mucosal surface [14]. Chitosan used in formulation is a natural cationic polysaccharide; it is also extensively reviewed in the literature. It has several uses within the pharmaceutical area due to its favourable characteristics such as non-toxicity, biocompatibility, biodegradability and some properties like bio-adhesion [15]. It is normally produced from chitin (the second most abundant polysaccharide after cellulose) through enzymatic degradation or alkaline deacetylation. Studies about bioadhesive properties of chitosan showed that this polymer had deep absorption promoting effects when administered nasally with polar molecules such as peptides and proteins in animal models [16].

In this study, a proposal for an affordable way to obtain the antigen and a promisor administration route for a vaccine against a disease acquired by respiratory tract is presented.

Materials and Methods

PsaA raw obtainment

The PsaA gene studied in this work was developed from Larentis and co-workers studies [8] (PsaA gene from *S. pneumoniae* serotype 14 – strain 1871 from SIREVA Project/Canada), and it was expressed in the *E. coli* BL21 (DE3) Star strain with IPTG induction of the plasmid pET28a (Novagen).

A pre-inoculum with 50μL of recombinant *E. coli* BL21 (DE3) Star cells was cultivated in 50 mL TB medium (Terrific Broth medium 24 g/L yeast extract, 12 g/L tryptone, 12.67 g/L K_2HPO_4, 2.31 g/L KH_2PO_4 0.4% glycerol) supplied with 50 μg/mL kanamycin and 1% glucose at 37°C for 16 h at 120 rpm in 250 mL Erlenmeyer flask. Three 1.4 mL aliquots from the resulting pre-inoculum were inoculated in three vessels of bench scale bioreactors (Fogale Biopod f800) with 70 mL of TB medium, containing 50 μg/mL kanamycin, 1% glucose and antifoam. Cultures were maintained at 37°C, with air lifting at 1vvmfor two hours, and after completing this time, 7 μL of 0.1mM IPTG was added in each vessel. Expression temperature was set at 25°C and for each vessel the time was adjusted to 16, 10 and 6 h in order to compare the PsaA productivity in different expression times. Cells were harvested by centrifugation at 4000rpm for 20 min at 4°C (3,220 g in Eppendorf 5810R centrifuge). Pellets were stored at -20°C.

Purification

Pellets were resuspended in 20 mMTris/1 mM EDTA (pH 8) according to the ratio of 5 mL buffer/g (wet mass) of pellet. The cellular suspension was disrupted by sonication on ice in5 cycles of 10 s pulses in 30 sec. intervals, 30% amplitude using an ultrasonic cell disruptor by Sonics & Material, Inc. Protein extracts were centrifuged at 18,514 g, 20 min at 4°C (Eppendorf 5810R centrifuge) and the soluble fractions were filtered using a 0.2 μm membrane to perform the purifications using Akta Pure system (GE Healthcare). Purification was performed separately with each pellet using a XK 16/20 column filled with 25 mL of DEAE Sepharose fast flow (GE Healthcare). Injection volumes were eluted at 5 mL/min, corresponding to 150 cm/h in linear flow, using stepwise gradient at 4%, 15% and 100% with 20 mMTris/1 mM EDTA (pH 8) as buffer A and 20 mMTris/1 mM EDTA (pH 8) 1M NaCl as buffer B. PsaA fractions were identified at 15% step. Purified fractions were collected to perform desalting using Sephadex G-25 (medium) with the XK 26/40 column (GE Healthcare) using PBS pH 8 buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4). The purified protein was stored in this buffer at -20°C.

Desalting using Tangential Cross Flow Filtration (TFF)

Alternatively, to the G-25 desalting step, a TFF was performed, in the first cultivation studies using a Sartocon Slice with a 10 kDa (cutoff) polyethersulphone membrane (PES, Sartorius). A volume of 279.6 mL (553.61 mg) of purified protein from an Erlenmeyer of 16 h cultivation was concentrated and diafiltrated by tangential cross flow filtration using PBS pH 8 buffer as exchange buffer. A 0.02 m^2 membrane was used in a ratio of 27.68 g protein per square meter. The final volume and concentration were measured to check process yield.

Formulation

Chitosan particles were prepared using a modified Berthold (et al.) precipitation-coacervation method [17]. Chitosan (Sigma, 80% deacetylation degree) solutions were prepared with 0.25, 0.5, 0.75 and 1.0% polymer mass with 1% (v/v) of acetic acid and 1% w/v of Tween 80. For each solution, 2.08 mL PsaA solution were dropped in 20% w/v sodium sulphate to obtain the final solution with approximately 20 μg protein/mL. Formulation was maintained in constant stirring (200-500 rpm) for 60 minutes at room temperature, and centrifuged for 30 min at 2300 g. Encapsulation efficiency was calculated indirectly by measuring protein concentration from the initial solution and from the supernatant after removing the encapsulated chitosan particles. Encapsulation efficiency was presented as follows:

((initial concentration – supernatant concentration) /(initial concentration))x 100.

Analytical methods

The quantification of total proteins was performed using the BCA protein assay kit (Pierce®) according to the manufacturer's instructions.

DNA content was determined using Qubit™ Quantification Fluorometer (Invitrogen Corp.), following the methodology described in the manufacturer's instructions.

Endotoxin content was determined using chromogenic method with Endosafe® system, following the methodology described in the manufacturer's instructions.

The homogeneity of the sample after purification step was verified by SDS-PAGE, using 4-12% Novex® NuPAGE® (Thermo Fischer Scientific). Electrophoretic running was realized in XCellSureLock System® (Thermo Fischer Scientific). After scanning, the gel was analyzed in ImageMaster® software (GE) for band quantification by densitometry. SDS-Page for final samples was performed using PhastGel Gradient (GE Healthcare) with polyacrylamide 12.5%. The used molecular weight standard was Precision Dual Color (BioRad).

The purified PsaA and formulated in chitosan were tested for identity and immunogenicity using western blot technique. The purified protein formulated in chitosan 0.25% were diluted in PBS pH 8 buffer and was incubated at 37°C for 4 hours. After this, the supernatant was quantified by BCA and it was used for western blot analysis with anti-rPsaA rabbit serum. The protein purified was also tested with western blot in order to verify the protein integrity before and after formulation process. The proteins were transferred to a nitrocellulose membrane (Bio-Rad) at 60V for 90 min. Membrane was blocked with PBS-skim milk (4%)-Tween 20 (0.05%)-BSA (Bovine Serum Albumine) (0.25%) overnight at 4°C. A primary antibody to rPsaA diluted 1:4000 in PBS-Tween 20 (0.05%)-BSA (0.25%) (PBSTB) was applied to the membrane and incubated for 2 h at room temperature. Membrane was washed four times for 10 min with PBS-0.05% Tween 20. Conjugates anti-rabbit

Igg alkaline phosphatase (Sigma) was then applied to membrane at a 1:30,000 dilution in PBSTB and incubated for 1 h at room temperature. Membrane was washed as described above, subjected to a final wash with PBS and revealed with Westrn Blue Stabilized Substrate for Alkaline Phosfatase (Promega). Densitometry of the gel was performed using Image Master 1D software.

Particular size of the formulations was determined using DLS (dynamic laser light scattering) technique: samples were submitted to sonication (to avoid particles aggregates), using UPS 2005-Hielschersonicator and diluted with purified water to reach 0.2 AU at 280 nanometers. After these procedures, the measurements were performed in a particle analyzer SALD2201 (Shimadzu).

Results

Expression times for obtaining raw PsaA

In order to make possible and more affordable to industrial scale, the reduction of expression time was considered and was one of the aims of this study. Figure 1-A represents the first step purification gel from samples provided from 16, 6 and 10 hours cultivation. It shows that expression levels can be considered the same, that is possible to be verified in Figure 1-B, presenting the densitometry of the lane 2 (16 hours, 68.1%), lane 3 (6 hours, 73.7%) and lane 4 (10 hours, 77.6%), without considerable changes of protein's profile and homogeneity. The bands revealed were all on 37 kDa, characteristic of PsaA.

Purification

In the ion exchange chromatography was observed homogeneity levels around 70% (Figure 1-B), and average in mass recovery of 34%, in a technique that is simple to perform and possible to be scaled up. As example, in Figure 2 presents a purification profile of the sample obtained from 10 hours of expression process. Table 1 presents the results from first step purification, using ion exchange chromatography.

For desalting, two techniques were tested separately in order to evaluate possibilities to be performed in industrial scale and the results are presented in Table 2. In the desalting step, the mass recovery was around 80-90% and purity levels are expressed in Figure 3, A-B.

In order to proposal a complete process to obtain purified PsaA in an affordable way to be performed in industrial scale, Table 3 summarizes purification results from initial purification and desalting, presenting a complete process for PsaA production.

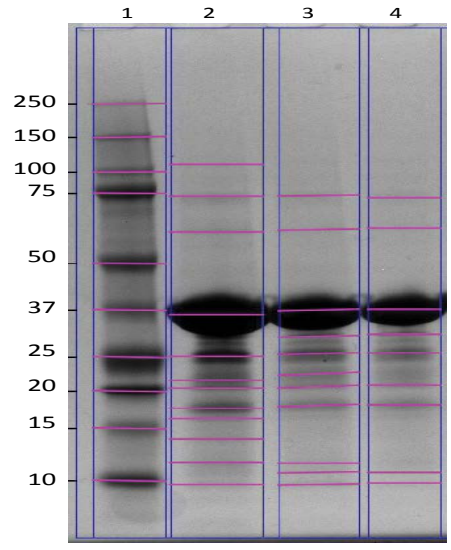

Figure 1-A: PreCasting Novex NuPAGE 4-12% denaturanting gel of samples from DEAE Sepharose purification. Lane 1: molecular weight standard. Lanes 2, 3, 4: extract from 16, 6 and 10 hours cultivation respectively, after purification step, at 15% elution gradient. Same mass protein was applied on the gel.

Formulation

Changes in chitosan concentration resulted in different encapsulation efficiency (Table 4).

Characterization and immunological identity

Table 5 presents size particle results from formulations samples of PsaA in each chitosan concentration.

Based on the better result in particle size, identity test was performed with 0.25% chitosan formulation. Characterization of immunological identity tests with this formulated antigen and purified PsaA were performed and positive identification were presented for purified PsaA and formulation (Figure 4). Density analysis of these tests is presented in Figure 5.

Discussion

High levels of expression of heterologous proteins can be achieved by using recombinant *E. coli*. However, as disadvantage, the presence of Lipopolysaccharide (LPS) on its surface, and the fact that heterologous protein accumulate intracellularly, forming in some cases insoluble aggregates named inclusion bodies, leads to include more steps along the purification process. In this case, studies have shown that a simple temperature reduction of bacterial growth can decrease the incidence of inclusion body formation, and optimize the engineered plasmid [18]. Besides, if is possible to perform shorter expression times, with similar yields, it is possible to reduce LPS production, DNA from the host, and other metabolites that must be removed during purification.

In first step of the purification (Table 1) was possible to obtain high levels of purity and DNA clearance, this is an important result for human applications. The capacity used in this process has an average of 4.6 mg of protein for each mL of resin DEAE Sepharose FF, and it is possible to reach better results in productivity with process adjusts as the DEAE's producer, indicates a dynamic bind capacity of 110 mg of a standard protein for each mL of resin [19]. From first step purification, we can observe that 10 hours expression process can be the better choice, as gives higher purity levels in PsaA and higher DNA clearance. Even with the bigger biomass productivity of 6 hours expression process, the productivity on PasA in 10 hours expression was 80% higher than PsaA productivity found in 6 hours expression. However, studies aiming to improve the performance of the 6 hours expression can be advantageous to reach a gain of 10 hours from the original 16 hours expression process.

In industrial scale, the pellet treatment with sonication must be replaced by homogenization process, a cell disruptor with pressurized flow and refrigeration to promote cell lysis followed by centrifugation in continuous flow equipment to recovery the soluble fraction.

In desalting step (Table 2), we studied gel filtration and tangential cross flow filtration. These two techniques and counter current dialysis are the three major methods for buffer exchange of proteins in industrial scale [20]. Comparing both techniques of desalting PsaA, the final protein is not diluted in TFF, and in GF it was diluted to the half from initial. Thus, it should be considered in industrial scale, because in this final step, the concentration is an important factor to planning formulation steps and in TFF, the final concentration and desalting can be adjusted in a same step.

In previous comparative studies, was observed that the amount of buffer required for TFF is about one-third of that required for GF; the plant space in TFF is about one-half of required for GF, and total operating time is one-half lower for TFF [20]. So, based on the information above, TFF could be a better choice for PsaA desalting,

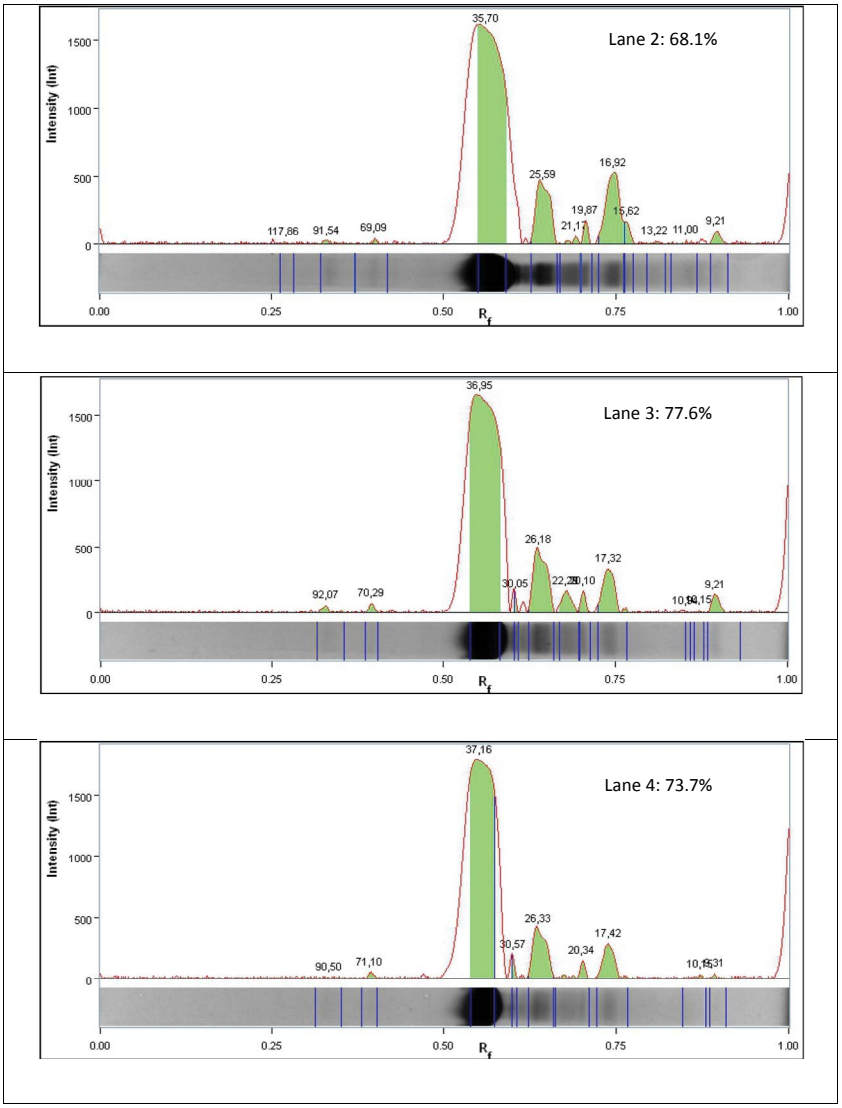

Figure 1-B: Densitometry analysis of the lanes 2 (16 h), 3 (6 h) and 4 (10 h) from PreCastingNovexNuPAGE 4-12% denaturanting gel of samples from DEAE Sepharose purification (Figure 1-A). The values in the lanes representing the PsaA homogeneity of each sample.

Initial protein				**Final protein**						
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Expression (h)	**Biomass (g)**	**Initial protein (mg/mL)**	**Initial protein (mg)**	**Final protein (mg/mL)**	**Final protein (mg)**	**Yield (%)**	**Purified fraction purity (%)**	**DNA clearance (%)**	**PurifPsaA (g)**	**RatioPsaA/g biomass**
16	2.23	24.18	120,9	3.015	45,23	37	68.1	15	0,031	0,014
10	1.88	24.96	124,8	2.986	44,79	36	77.6	98.8	0,035	0,018
6	2.06	19.74	98,7	2.434	29,21	30	73.7	89.9	0,022	0,010

Columns legend:
1: Time expression of each bench-scale bioreactor
2: Biomass obtained in each bioreactor
3: Initial protein concentration of soluble fraction before purification
4: Total protein initial in 5 mL of injected volume
5: Final protein concentration in purified fraction
6: Total protein in purified volume
7: Yield considering the ratio between initial mass and purified mass of total protein
8: Purity considering densitometry analysis (Figures 1-A and 1-B)
9: DNA clearance considering initial and final DNA content of each sample
10: g of PsaA in purified fraction, calculate by final protein (mg) in column 6 versus purity in column 7
11: Ratio of PsaA production, considering g PsaA generated for 1 g of biomass (as higher is this value, more PsaA is obtained)

Table 1: Results from experiments from first step purification (DEAE Sepharose™ FF).

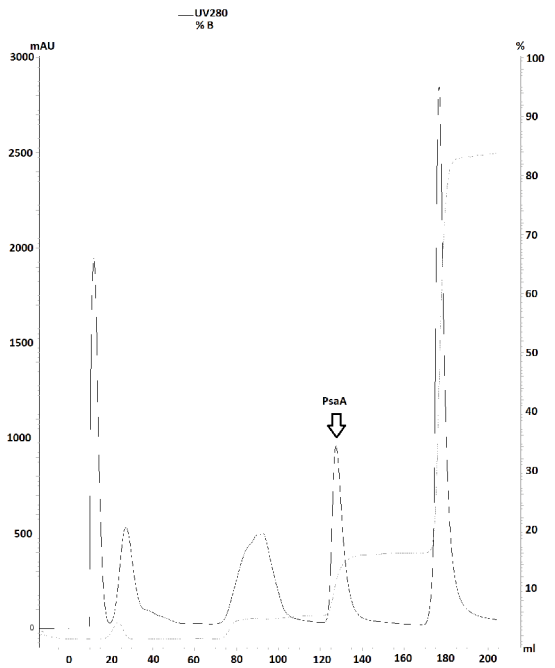

Figure 2: Stepwise gradient elution of PsaA 10-h expression in DEAE Sepharose FF. Injection volume: 5 mL, flow rate 5 mL/min. Elution was carried out by increasing [NaCl] in Tris /EDTA buffer (pH 8). Detection UV 280 nm.

as were not observed alterations in SDS-PAGE profiles (Figure 3), suggesting that pump circulations in TFF process did not cause changes in the results. Better recoveries in TFF can be achieved implementing a washing step at the end of the process.

As presented, DNA contents (0.39 ng/dose) are in accordance to the recommendation of the World Health Organization (< 10 ng/dose) [21]. In order to obtain this results, one dose was considered as 15 micrograms of purified PsaA, based on previous studies from Xu and co-workers (2015) [22]. Endotoxin levels presented different results considering TFF and gel filtration: for gel filtration, we found 68.25 EU/dose and TFF, 4.93 EU/dose. Considering one dose with 15 micrograms [22] and 1 mL as administration volume, we achieved satisfactory endotoxin levels results using TFF, according to Brito and Singh work

Desalting technique	G-25	TFF
Initial volume (mL)	15	279,6
Total protein initial (mg/mL)	5,25	1,98
mg initial	78,75	553,61
Final volume (mL)	35	205,91
mg/mL final	2.08	2.19
mg final	72.8	450,94
Yield (%)	92,4	81,4
Endotoxin (EU/dose)*	68.25	4.93

* considereng 1 dose with 15 micograms of PsaA [22]; EU: endotoxin units

Table 2: Desalting results in different techniques.

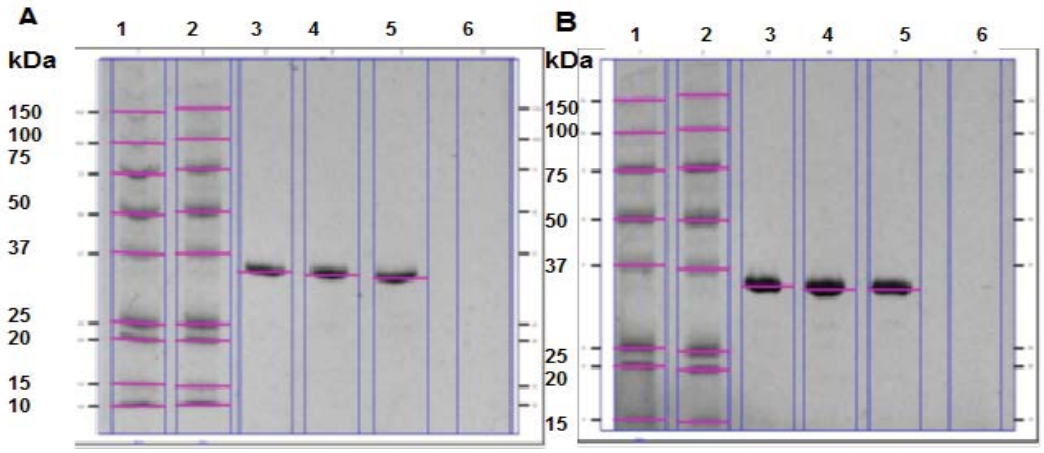

Figure 3: PhastGel polyacrylamide 12% denaturanting gel. A: Sample from gel filtration desalting. Lanes 1-2: molecular weight standard (duplicate). Lanes 3-4-5: samples after desalting (triplicate). Lane 6: PSB buffer. B: Sample from desalting tangential cross flow filtration.

First step purification results (DEAE Sepharose™)				
Expression (h)		**yield (%)**	**Homogeneity (%)**	**DNA clearance (%)**
10		36	77.6%	98.8
Second step purification – Desalting				
Technique	**yield (%)**	**Homogeneity (%)**	**Endotoxin (EU/dose)****	**DNA (ng/dose)****
Tangential flow filtration	81.4	> 99	4.93	0.39
Overall mass protein yield 1: 29%*				
*Considering initial mass from 10 hours expression (124.8 mg) and 36.04% recovery from ion exchange chromatography (IEX) and 81.4% recovery from TFF desalting				
**Considering 1 dose with 15 micrograms of PsaA [22]				
Yield was calculated by the ratio: (mass of total protein x 100)/ (mass in previous step)				
EU: endotoxin units				

Table 3: Results from process proposal for obtainment of purified PsaA, considering 10 hours expression, first step of purification using DEAE Sepharose™FF and desalting using tangential cross flow filtratiom (polyethersuphone membrane, pore 10 kDa, 0.02 m^2).

Chitosan (%)	Encapsulation efficiency (%)
0.25	11.8 ± 1.3
0.50	21.0 ± 6.6
0.75	33.3 ± 1,6
1.0	50.6 ± 0,78

Table 4: Encapsulation efficiency with different chitosan concentrations. Average from two experiments with standard deviation.

Chitosan (%)	Size particle (µm)	Standard deviation
0.25	0.718	0.158
0.50	1.068	0.294
0.75	1.333	0.300
1.0	1.086	0.324

Table 5: Size particle results for formulations by DLS.

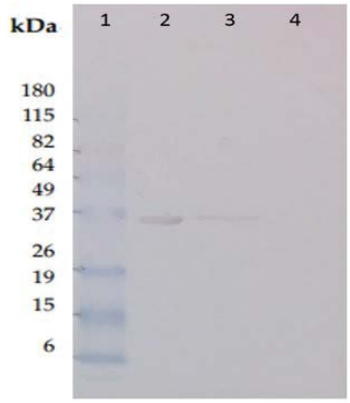

Figure 4: Western blot from purified PsaA and formulated in chitosan 0.25%. Lanes: 1-BenchMark™ Pre-Stained Protein Standard; 2-PsaA; 3-PsaA formulated in chitosan 0.25%; 4-Nacked Chitosan 0.25%formulation.

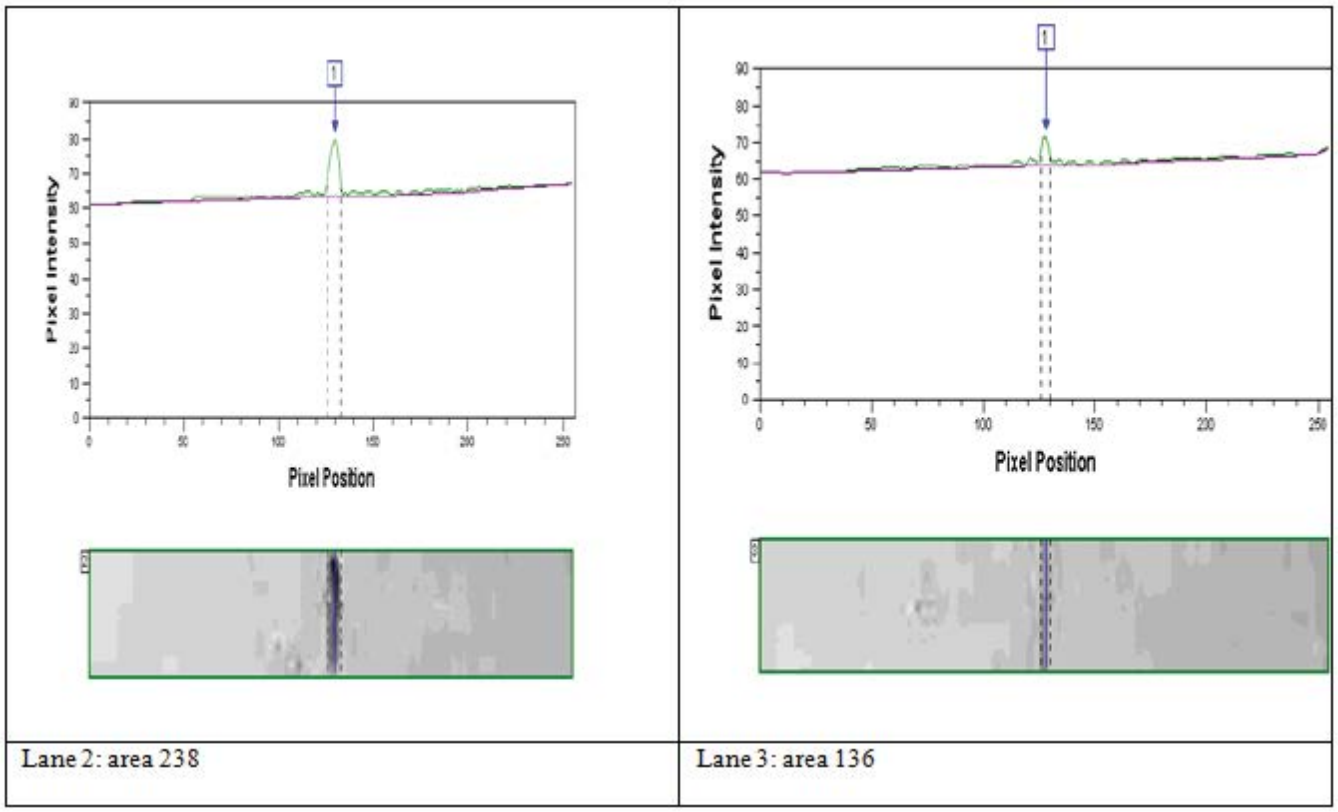

Figure 5: Densitometry analysis from lanes generated on western blot gel (Figure 4). Area of the bands was calculated using Image Master 1D software. Lane 2: standard PsaA; Lane 3: 0.25% chitosan PsaA formulation. Lane 4 (nacked chitosan) did not presented detectable signal.

(2011), which considers 20 EU/mL as limit for recombinant subunit vaccine [23]. From these results, it's possible to consider TFF as a better choice in terms of endotoxin clearance efficacy, as it's was capable to select more precisely the purified protein by molecule size. In order to improve the security of the process, it's possible to use detergent into TFF desalting buffer to assure the endotoxin removal.

In chitosan formulation proposal, better encapsulation efficiency was observed for sample using 1.0% chitosan formulation (Table 4). However, at higher chitosan concentration, more difficulties in preparing solutions and resuspending the formed particles were observed. Previous studies described experiments using chitosan concentration up to 1% (w/v) [3,13,24,25], but these are solution with some particularities in correct salt concentration and higher grades of chitosan generates aggregates formation, which was observed in size particles results (Table 5).

Particle size considered optimal for uptake by dendritic cells and macrophages could be defined as 1 µm, but Antigen Presenting Cells (APCs) can be better activated using 0.3 μm chitosan particles [24]. Therefore, from these experiments 0.25% chitosan was the better result in particle size, because was the result below 1 µm. Figure 4 presents the activity result of this formulation and it's possible to verify positive identity for PsaA, even with lower intensity, comparing

with PsaA standard used. It is because chitosan formulations present several difficulties in sample treatment for *in vitro* tests. As the 0.25% formulation presented lower encapsulation efficiency, other tests using chitosan in better conditions (charge, pH and functionalized chitosan) will be performed to reach better encapsulation results and to be used in identity tests before to start animal tests. Most of previous studies with chitosan formulations did not presented *in vitro* tests, going directly to animal tests. Animal tests are crucial to efficacy results, but it is also important present first a robustness on identity tests and other safety *in vitro* tests with the formulation (Endotoxin, DLS, stability) in order to grant an industrial scale pharmaceutical process.

Conclusion

In this work it is possible to verify that a recombinant vaccine could be feasible to use in pneumococcal vaccine, because of the proposed process can reach high productivity in fermentation step in an affordable way to obtain this antigen as unique active ingredient, in opposite to several fermentations, purification steps and couplings, as happens nowadays. Satisfactory results of endotoxin and DNA were presented for active pharmaceutical ingredient using simple and scalable purification steps.

In addition, an intranasal formulation with chitosan was proposed, an accessible polymer as an alternative to syringe and needles that could promote more adhesion of children and elderly, which are considered groups in risk of pneumococcal pneumoniae disease. In formulation step, other tests must be performed with functionalized chitosan and other salts concentrations granting ionic stability to reach better encapsulation and particle size rates, enabling the formulation to identity tests and animal tests.

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