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A chitosan-based nanosystem as pneumococcal vaccine delivery platform

Sandra Robla, Maruthi Prasanna, Rubén Varela Calviño, Cyrille Grandjean & Noemi Csaba.

Abstract

Chitosan-based nanosystems have been described as interesting tools for antigen delivery and for enhancing the immunogenicity of nasally administered vaccines. As a possible vaccine delivery method, the chemical conjugation of chitosan nanocapsules with the *Streptococcus pneumoniae* cell membrane protein PsaA (pneumococcal surface adhesin A) is suggested here. The antigen PsaA, common to all pneumococcus serotypes, is expected to improve its uptake by immune cells and to activate specific T cells, generating an adaptive immune response against pneumococcus.

With this aim, chitosan nanocapsules with thiol-maleimide conjugation between the polymer (chitosan) and the antigen (PsaA) were designed to enable the surface presentation of PsaA for immune cell recognition. Spherical shaped particles, with a size of 266 ± 32 nm, positive charge of $+30\pm 1$ mV and good stability profiles in simulated nasal fluids (up to 24 h) were achieved. PsaA association rates were three times higher compared to nanocapsules without covalent polymer-protein conjugation. Cytotoxicity studies in cell culture media showed non-toxic effect under $150\text{ }\mu\text{g/mL}$ concentration of nanocapsules, and subsequent studies on the maturation of immature dendritic cells in the presence of antigen-conjugated nanocapsules displayed peripheral blood mononuclear cell activation and lymphocyte differentiation after their presentation by dendritic cells. Secretion of TNF α following exposure to nanocapsules and the ability of nanocapsules to activate CD4 and CD8 T lymphocytes has also been studied.

Graphical abstract: Antigen loaded nanocarriers uptake and presentation by professional presenting cells.

Keywords: Chitosan, nanovaccine, dendritic cell, antigen protein, immune response.

1. Introduction

Streptococcus pneumoniae is an opportunistic pathogen that colonizes the human upper respiratory tract and causes pneumonia, meningitis and septicemia [1]. It is responsible for high morbidity and mortality rates, especially among infants, elderly and immunocompromised people [2]. The cell surface of *S. pneumoniae* contains various proteins and capsular polysaccharides (CPS) that play an important role in pathogen attachment and immune system evasion. The CPS diversity results in more than 96 stereotypes whereas cell surface proteins are more conserved and less variable [3]. Currently available CPS-based vaccines such as PCV13 and PCV10 (Pneumococcal Conjugated Vaccines) and PPSV23 (Pneumococcal Polysaccharide Vaccine) only induce specific serotype dependent immune responses [4]. In addition, PPSV23 lacks the ability to generate robust immune responses in high-risk populations such as infants or the elderly. To address some of these obstacles, several studies investigated the possibility of using protein antigens expressed at the *S. pneumoniae* cell wall as potential vaccine candidates. One relevant cell membrane protein is Pneumococcal surface adhesin A (PsaA) [5]. PsaA is a critical factor for the colonization and adhesion of the pneumococcus in nasopharyngeal epithelial cells. It has been shown to activate both the humoral and cellular response of the immune system against the entry of the pathogen via the respiratory airways [6].

Intranasal route has been extensively explored for non-invasive immunization, stimulating mucosal and systemic immunity and protecting against respiratory infections [7]. The protective nasal epithelial barrier presents a large surface area which produces a rapid permeation of drugs [8], as well as the NALT (Nasal Associated Lymphoid Tissue), responsible for the production of long-term antigen-specific antibodies [9][10]. The nasal barrier exhibits metabolic activity and is covered with a dynamic mucus layer which limits the permeability of the epithelium and decreases formulation residence time [11]. In this context, nanostructures have been described as interesting tools to enhance the delivery of proteins, improving the immunogenicity of nasally administered vaccines [12]. Indeed, nanostructures play an important role in the stimulation of an adaptive immune response, upon facilitating the antigen uptake and processing by professional antigen-presenting cells such as dendritic cells (DCs) [13], and the immediate innate immune response against these inhaled external substances by macrophages [14]. Chitosan (CS), a polysaccharide derived from the deacetylation of chitin [15] has been widely used in the design of nanocarriers for nasal absorption of peptides like insulin, salmon calcitonin, leuprolide or parathyroid hormone [16], thanks to its high mucoadhesiveness, biocompatibility and biodegradability [17]. Previous studies have shown that chitosan nanocapsules are good candidates for protein administration by nasal route [16], and also have been reported to have an adjuvant effect by promoting innate immune responses [18]. In this research, a mucoadhesive chitosan nanocarrier containing a covalently conjugated pneumococcal protein antigen was designed and characterized to evaluate its capacity as a nasal drug delivery system..

2. Materials and methods

Miglyol 812® was purchased from Cremer Oleo (Hamburg, Germany), polyethylene glycol stearate 40 (PEG-st 40) was purchased from Croda Chemicals Europe Ltd (Snaith, United Kingdom) and sodium glycocholate (SGC) was obtained from Dextra laboratories Ltd (Reading, United Kingdom). Chitosan HCl ((C₆H₁₁NO₄Cl)_n, Mw: 1526.5 g/mol, deacetylation degree of 85%) was purchased from Heppe Medical Chitosan GmbH (Saale, Germany). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, C₈H₁₇N₃, Mw: 191.7 Da), *N*-Hydroxysuccinimide (NHS, C₄H₅NO₃, Mw: 115.09 Da), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride hydrate (CaCl₂·2H₂O) and mucin type III were purchased from Sigma Aldrich (MO, USA). Maleimide propionic acid 95% (m-PA, C₁₁H₁₀N₂O₆, Mw: 169.13 Da) and hydroxylamine were obtained from Alfa Aesar (MA, USA). Cyanine5 NHS ester dye (Cy5-NHS) was provided by Lumiprobe (Hannover, Germany), S-acetylthioacetate succinimidyl ester

(SATA, Mw: 231.23 Da) and the SnakeSkin™ Dialysis Tubing 3.5K MWCO were obtained from Fischer scientific (MO, USA). The Ni-NTA resin was obtained from Macherey-Nagel, and PsaA used in the experiments was provided by UFIP UMR CNRS 6286 at University of Nantes.

For *in vitro* experiments sterile and autoclaved material were used. Macrophage Raw 264.7 cell line was obtained from ATCC (VA, USA), Ficoll-Paque™ PLUS (density 1.077 g/mL) was purchased from GE Healthcare Bio-Science AB (IL, USA), Roswell Park Memorial Institute medium (RPMI-1640) and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from GIBCO® (Thermo Fisher Scientific, MA, USA). Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 4 (IL-4), allophycocyanin (APC)-conjugated anti-human CD83 (CD83-APC), phycoerythrin (PE)-conjugated anti-human CD86 (CD86-PE) and fluorescein (FITC)-conjugated anti-human CD1a (CD1a-FITC), were purchased from Miltenyi Biotec (CA, USA), anti-human CD4-APC, CD8-APC; CD25-PE and CD28-PE were obtained from Tonbo Biosciences (CA, USA). Fluorescein (FITC)-conjugated anti-human CD3 (CD3-FITC) and phycoerythrin (PE)-conjugated anti-human Tumor Necrosis Factor alpha (TNF-α) were both from Miltenyi Biotec (Madrid, Spain). Fetal Bovine Serum (FBS), PSG (100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine) and Ebioscience™ 7-aminoactinomycin D (7-AAD) viability staining were purchased from Invitrogen (CA, USA). Lipopolysaccharide (LPS) from *Escherichia coli* and Brefeldin A (BFA) were purchased from Sigma Aldrich (MO, USA). MTS (reactive de Owen (3-(4,5-dinitiltiazol-2-yl)-5(3-carboximethoxyphenyl)-2-(4-sulfofenil)-2H tetrazolium) cell proliferation assay kit was provided by BioVision (CA, USA). Triton® X-100 was purchased from Scharlab (Barcelona, Spain), and interferon gamma (IFNγ) was purchased from Peprotech, (London, UK). All other chemicals were of reagent grade or higher purity.

2.1. Antigen production and characterization

The mature PsaA used as an antigen was previously expressed and purified [19]. Briefly, a plasmid encoding PsaA deprived of its signal peptide was transformed into *E. coli* BL 21 cells. Fractions containing the target protein were purified by affinity chromatography and the protein was purified to eliminate any traces of endotoxin present using endotoxin removal beads.

2.2. Functionalization of the polymer and proteins and their characterization

2.2.1. PsaA-SATA conjugation

For functionalization of PsaA, 1.35 mg of SATA was incorporated at 45 minute-intervals for 3 times (0.45 mg x 3, ~40 equiv) into 5 mg/mL of PsaA antigen in PBS 0.1 M pH 7.4, at 4°C under stirring. Subsequently, the reaction mixture was dialyzed in 0.1 M PBS to eliminate the excess of reagent using dialysis membrane (3.5 kDa) at 4°C. The ability of the conjugated protein to retain its structural conformation was studied using circular dichroism (CD) using Jasco J-810 spectropolarimeter. The baseline was obtained using a blank solution containing the buffer. Solution of PsaA or the conjugated PsaA-SATA was diluted to 0.1 mg/mL in buffer solution and was scanned over the wavelength range 200–260 nm. The analysis was performed using a 1-nm quartz cylindrical cell at a scanning speed of 50 nm/min, 0.1 nm bandwidth and a temperature of 20°C. PsaA and PsaA-SATA mass spectra were also determined on an Autoflex III MALDI-TOF/TOF spectrometer (Bruker Daltonics).

2.2.2. Chitosan-maleimide conjugation

In brief, maleimide-propionic acid (0.553 mg, 5:1 ratio CS:m-PA) in dimethyl sulfoxide (DMSO) was reacted with 5 mg of chitosan in the presence of 0.339 mg of NHS (2:1 ratio CS:NHS) and 0.188 mg of EDC (2:1 ratio CS:EDC) as crosslinkers and were kept under constant stirring for 24 h. Excess reagents were removed by dialysis using dialysis membrane (3.5 kDa). The chemical conjunction between chitosan and maleimide-propionic acid was confirmed by ^1H -NMR spectrometry (Bruker BRX-500).

2.3. **Preparation of the nanocapsules**

Blank CS and CS-maleimide nanocapsules (NCs) were prepared by solvent displacement technique [20]. An organic phase of 1.6 mg of PEG st-40, 0.5 mg of SGC and 6.25 μL of Miglyol[®] (density 0.94 g/mol) in a final volume of 500 μL of ethanol was poured over a 0.5 mg/mL CS (for CS NCs obtention) or CS-maleimide (for CS-maleimide NCs) solution [21], and was kept under continuous stirring for 1 h. Later, the organic solvent was removed under vacuum using rotary evaporation (R-300 Buchi[®], 150 rpm, 50 mbar, 5 min at 37°C) to a constant volume of 1 mL. Finally, nanocapsules were isolated by centrifugation (Eppendorf 5430R[®], at 20.000 g for 30 min at 4°C) and resuspended in ultrapure water.

The association of PsaA and PsaA-SATA was carried out at the end of the synthesis of CS and CS-maleimide nanocapsules, respectively, using a mass ratio of 1:5 (PsaA:polysaccharide) for the association of the protein. In both cases, protein was added and kept under continuous stirring for 10 min, and to ensure the entire removal of unassociated PsaA/PsaA-SATA, nanocapsules were centrifugated (10.000 g for 5 min at RT).

For *in vitro* studies, the conjugated NCs were labeled with the fluorescent marker Cy5. For this, Cy5-NHS in DMSO was added to CS-maleimide in acetate buffer at pH 5.6 (mass ratio 1:1) and kept overnight under magnetic stirring. Then, the reaction was stopped, and excess dye was removed using PD10 columns (Centri-Pure P10- Gel Fixing Column, Zetadex-emp Biotech GmbH, Berlin, Germany) and the purified product was dialyzed again overnight.

Optionally, NCs were freeze dried in the presence of trehalose 5% (w/v) as cryoprotectant. NCs and trehalose solution were mixed 1:1 (v/v) in a freeze-drying glass vials, they were frozen at -80°C and then transferred to the freeze-drier (Labconco Corp., USA), using a primary drying cycle from -40°C to 20°C (+10°C/step, 18h, 12h, 5h and 4x2h) and a secondary drying cycle (22°C, 3 h and -20 mTorr vacuum conditions).

2.3.1. Physicochemical characterization of nanocapsules

The hydrodynamic diameter, polydispersity index (PDI) and derived count rate of the prepared NCs were measured using dynamic light scattering (DLS). The zeta potential values were obtained by laser dropper anemometry using a Zetasizer Nano-S (Malvern Instruments; Malvern, UK). The NCs were diluted 1:20 in ultrapure water (MilliQ®, Merck, NJ, USA) prior to the measurement. Analysis was performed in triplicate at 25°C with a detection angle of 173° in ultrapure water. Nanoparticle tracking analysis (NTA) was used to measure the particle size of NCs based on imaging after dilution in ultrapure water using a NanoSight NS3000 equipment (Malvern analytical Ltd., Malvern, UK). For NTA analysis CS NCs were diluted 1:100 to keep the concentration within the instrument measuring range.

2.3.2. Association efficiency

The association efficiency (AE%) of protein was estimated by quantifying the difference in absorbance of the total amount of protein and the amount of free protein present in the supernatant after centrifugation. The free protein in the supernatant was analyzed by Micro BCA assay colorimetric method (Micro BCA Protein Assay, Thermofisher Scientific, Spain) using a microplate reader (Synergy H1 Hybrid Multi-Mode, BioTek, Winooski, VT) by measuring the absorbance at 562 nm.

2.3.3. Morphological characterization

The surface morphology of the nanocapsules was studied using FESEM and STEM electron microscopy (FESEM Ultra Plus, ZEISS, Germany). For FESEM studies, 10 µL of the diluted sample was placed on the silicon wafer and kept in the desiccator overnight for drying. Before the analysis, the samples were sputter

coated with iridium in an argon atmosphere. For STEM studies, NCs were also diluted 1:100 and 10 μ L was placed on copper grids with carbon films and then washed drop-by-drop with water. Later the grids were air dried overnight in the desiccator and the analysis was performed.

2.3.4. Colloidal stability

The colloidal stability of the antigen loaded nanocapsules before and after freeze drying procedure was studied *in vitro* using Simulated Nasal Fluid (SNF), composed of 7.45 mg/ml of NaCl, 1.29 mg/ml of KCl and 0.32 mg/ml of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and pH adjusted at 6.5. SNF supplemented with mucin type III (1% sialic acid content) at 0.1% was also used for the study. For further studies in dendritic cell culture, the stability of the nanosystems was assessed in cell culture RPMI-1640 medium, following the change in particle size, mean count rate and PDI over a period of 24 h. In brief, 20 μ L of NCs were mixed with 980 μ L of SNF with 0.1% mucin or 980 μ L of RPMI, while nanoparticles suspended in SNF and water at same concentrations are used as controls. The NCs in the different media were shaken at 100 rpm at 37°C using a constant-temperature shaker, at different time points (0, 6 and 24 h) the size and count rate of samples were measured using DLS as described in Section 2.3.1.

2.4. **Release studies**

In vitro release studies of PsaA from the conjugated nanocapsules was performed by incubating non-conjugated CS-PsaA and conjugated CS-maleimide PsaA nanocapsules in a final volume of 1 mL of SNF in a shaking incubator (Biosan Thermo-Shaker TS-100C, 600 rpm at 37°C). At different time intervals (1, 3, 5, 8, 12 and 24 h), samples were centrifuged (5 min at 10,000 g and RT) and 250 μ L of supernatant were collected for analysis and replaced with fresh SNF medium to maintain sink conditions [22]. The amount PsaA contained in the supernatant at scheduled time intervals were subsequently analyzed by absorbance measurement in a multilabel plate reader using BCA assay at 562 nm.

2.5. **Blood samples and dendritic cell preparation**

Heparinized blood samples were obtained from healthy donors after informed consent. The *buffy coats* from anonymous donors were donated by the Organ and Blood Donation Agency (ADOS; Santiago de Compostela, Spain). The peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll gradient centrifugation method [23]. Briefly, blood was diluted with PBS (1:1) and layered onto Ficoll-Paque™ PLUS at a ratio of 2:1 (blood:Ficoll). Different components were isolated after centrifugation (Allegra X-12R, Beckman Coulter) at 400 g for 30 min at RT, and PBMCs were collected from the interface, washed

twice with PBS and centrifuged again (300 g, 10 min at RT). Cells were resuspended in R₂ medium (RPMI-1640 supplemented with 2% heat-inactivated FBS and 1% of PSG). The density was adjusted to 1×10^6 cells/mL and 10 mL of cells were seeded into 75 cm² cell culture flask for 2 h (37°C, 5% CO₂). After the incubation period, non-adherent cells (peripheral blood lymphocytes or PBLs) were washed with PBS and attached monocytes were cultured for another 6 days, renewing half of the medium after three days, in R10 medium (RPMI-1640 supplemented with 1% antibiotics and 10% FBS) in the presence of IL-4 and GM-CSF (both at 100 ng/mL) to allow the cells to differentiate into DCs [24][25].

2.6. NCs toxicity on immune cells

2.6.1. MTS assay

The cytocompatibility of the antigen loaded CS-maleimide PsaA NCs with the dendritic cells was studied using MTS assay, and the presence of the soluble formazan product was determined by measuring the absorbance that is directly proportional to the number of viable cells [26]. Monocyte differentiated immature DCs (iDCs) were added into 48-well microplate (1×10^6 cells/mL) and blank CS-maleimide and CS-maleimide PsaA NCs with a concentration range from 400 µg/mL to 25 µg/mL were incorporated. As a negative control (0% toxicity), iDCs in culture medium was used, whereas, for the positive control (100% toxicity), iDCs were incubated with Triton X-100 (0.5%). In the same manner, macrophage Raw 264.7 cells were incubated with blank CS-maleimide and CS-maleimide PsaA NCs and treated using the same procedure as iDCs. After 4 and 24 h of incubation under standard sterile conditions for cell culture (37°C and 5% CO₂), cells were collected and centrifuged (Microfuge 5427-R, Eppendorf, 500 g, 5 min at RT) for the removal of un interacted nanocapsules. Finally, the cells were incubated with 10 µL of MTS reagent and the absorbance was measured at 490 nm using a microplate reader.

2.6.2. 7-AAD assay

Cytotoxicity studies on the dendritic cells were also carried out using the intercalative DNA fluorescent dye 7-AAD. iDCs were incubated for 24 h with different CS-maleimide PsaA NCs concentrations from 900 µg/mL to 50 µg/mL and after the incubation period, cells were washed and stained with 0.05 µg/µL of 7-AAD (30 min at 4°C) for analysis by flow cytometry (BD FACSCalibur™, Becton Dickinson). The emission of 7-AAD was detected in the FL-3 channel at 650 nm. The analysis was performed with Flowing software (Cell Imaging Core, Turku Biotechnology Center, Turku, Finland) [27].

2.7. Interaction of NCs with immune cells

The analysis of the uptake of CS-maleimide PsaA NCs by the DCs and Raw 264.7 cells was processed by means of confocal laser scanning microscopy (CLSM) and FESEM microscopy, respectively. For CLSM, DCs were incubated with Cy5-labeled nanosystems at a concentration of 150 µg/ml and they were visualized by confocal microscopy (Leica SP5, Mannheim, Germany) after 24 h. For FESEM analysis, Raw 264.7 cells were seeded on a poly-L-Lysine coated cellware (Corning Life Sciences, MA, USA) and nanocapsules were added, and allowed to incubate for 24 h. Later, the cells were fixed using 4% paraformaldehyde (PFA), washed and gradually dehydrated using increasing ethanol concentrations (30%, 70%, 96%, 100%). Cells were placed in the desiccator overnight and were sputter coated with iridium before visualization [26].

2.8. Human DCs phenotype analysis

iDCs were incubated with the conjugated CS-maleimide PsaA NCs at a final concentration of 150 µg/ml during 48 h. Cells were washed twice (400g, 6 min at RT) to eliminate excess NCs, resuspended in PBS and stained with optimal amounts of different antibodies (CD1a-FITC, CD83-APC and CD86-PE) for 25 min at RT, in the dark. Finally, levels of maturation markers were quantified by flow cytometry.

2.9. NCs tolerogenic effect in immature DCs

2,3-Indoleamine dioxygenase (IDO) expression was evaluated in iDCs maturation after conjugated CS-maleimide PsaA NCs exposition. In brief, 1.25 µl of L-tryptophan (100 µM) was added to the iDCs medium during the last 4 h of incubation. Then, cells were centrifuged (1,000 g, 5 min at RT) and supernatants were mixed with 30% trifluoroacetic acid (2:1 v/v) and centrifuged again (10,000 g, 5 min, RT). The supernatant was mixed with the Ehrlich Reagent (1:1 v/v) and absorbance at 490 nm was determined using a microplate reader as indicated.

2.10. Activation of CD4 and CD8 T cells by NCs

After total monocyte differentiation into iDCs, they were harvested, resuspended in R10 medium and iDCs (1×10^6 cells/mL) were co-incubated with 150 µg/ml of blank CS-maleimide NCs, CS-maleimide PsaA NCs and PsaA. As a negative control (no activation), iDCs in culture medium were used, whereas for the positive control (total activation), mature DCs (mDCs) were obtained by incubation of iDCs with bacterial LPS (20 ng/ml) and IFN-γ (200 IU/ml). After 48 h, cells were harvested and centrifuged to remove nanocapsules (300 g, 10 min at RT). In parallel, allogenic PBLs were obtained from *buffy coats* after 2 h of PBMCs incubation with R2 medium and they were co-incubated with DCs for 12 days, renewing culture media and

splitting the cells as necessary (usually every 2-3 days). Non-adherent cells were collected and centrifuged (500 g for 5 min at RT), washed and divided into two different groups. CD4-APCs together with CD25-PE (5 μ L/each) were added to the first, while CD8-APCs together with CD28-PE (5 μ L/each) were added to the latter and left to incubate at 4°C for 25 min in darkness. After incubation, cells were washed and centrifuged (400 g for 6 min at RT) for their analysis by flow cytometry as described above.

2.11. Cytokine secretion by PBMCs incubated with different NCs

PBMCs (1×10^6 cells/mL) were collected from healthy donors and incubated with blank CS-maleimide NCs, CS-maleimide PsaA NCs and PsaA alone (150 μ g/mL) (5% CO₂, 37 °C) for 2 h. After that, cells were washed and centrifuged (300 g for 10 min at RT), thus removing the nanocapsules. Then, BFA (10 mg/ml, 5 μ L/well) was added to block the secretion of cytokines and kept for overnight incubation. The following day, cells were centrifuged again under the same conditions, the PBMCs surface was stained with CD3-FITC (10 μ L/well) antibody and left for 15 min at 4°C in darkness. Cells were washed, centrifuged (400 g, 6 min at RT) and fixed with 1% PFA under constant agitation to avoid cell aggregation, and were incubated again for 15 min in darkness. Cell membrane was permeabilized with 1% saponin and centrifuged again under the initial conditions for an intercellular staining with TNF α -PE cytokine antibody (5 μ L/well) and incubated for 15 min at 4°C in darkness. Finally, cells were washed twice with 0.1% saponin buffer and fluorescence signals were measured by flow cytometry as indicated.

2.12. Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD) and mean $\pm$ standard error mean (SEM) for dendritic cells studies. Data analyses were carried out using GraphPad Prism v.6.01 software (GraphPad Software Inc., San Diego, CA, USA). Statistical analyses using the 2-way ANOVA test in combination with Dunnett's multiple comparisons test were used to determine the difference between donor groups.

3. Results and discussion

3.1. Functionalization of the polymer and protein and their characterization

3.1.1. PsaA-SATA conjugation

In the search for a serotype-independent pneumococcal vaccine, the use of highly conserved pneumococcal protein antigens has become an attractive alternative to achieve immune response against *S. pneumoniae* [28]. PsaA, a 35-kDa lipoprotein present in all serotypes known to date, is capable of inducing herd

immunity against the pathogen [29], and studies of its co-administration with PCV7 have demonstrated to increase serotype coverage and immunogenicity of PCV7 by reducing nasopharyngeal carriage [30].

In our study, the PsaA was obtained by rDNA technology and subsequently, the free amines present in the lysine amino acids of the PsaA were derivatized to produce a thiolated protein. The functionalization with SATA adds sulfhydryl groups to proteins in a protected form. Thus, PsaA when modified with SATA can be stored for extended time periods and the sulfhydryl groups required for the conjugation reactions will be exposed by treating with the hydroxylamine. Conjugation reaction between the PsaA protein and the SATA group is described in **Supplementary Figure 1a**. The MALDI-TOF technique (**Figure 1a**) used to determine the protein molecular weight showed that the purified PsaA had a mass of 34,059 Da, while PsaA-SATA conjugated protein had a mass of 35,683 Da. The difference in the molecular weight between PsaA and PsaA-SATA confirms the successful conjugation of SATA to PsaA antigen in the ratio of 13:1. The presence of multiple sulfhydryl groups on the protein helps its efficient coupling to the nanocapsules. Similarly, Derksen et al., have described the use of protein functionalized with SATA for its surface conjugation to liposomes containing maleimide residues [31]. In addition to this, the sulfhydryl modified proteins were also used to prepare protein-protein conjugate nanoparticles to enhance the immunogenicity of the protein antigens [32]. The conformation of the protein antigen is pivotal in conserving the potential epitopes that are responsible for the immunogenicity [33]. The CD spectra (**Figure 1b**) revealed no changes in the secondary structural conformation of the PsaA-SATA compared to the existing literature [19]. The PsaA antigen presented negative peaks at 208 and 221 nm, which were also observed in the conjugated PsaA-SATA antigen, indicating a similar protein folding pattern of alpha helixes and beta sheets in both cases [19,34].

Fig. 1: Pre-formulation characterization of PsaA and its conjugate PsaA-SATA: **(a)** graphs of MALDI-TOF for PsaA (blue line) and PsaA-SATA conjugate (green line). **(b)** Overlay of CD spectra for PsaA and PsaA-SATA conjugate.

3.1.2. Chitosan-maleimide conjugation

Chitosan is an attractive biomaterial with amine groups for conjugation of ligands [35], and previous studies have reported its successful covalent conjugation with maleimide groups [36]. Maleimide selectivity and efficient cross-linking with thiol groups, via Michael addition, has allowed the bioconjugation of chitosan with biomolecules such as peptides [37] or nucleic acids [38], avoiding the generation of reaction by-products. In the present work, conjugation between CS and maleimide propionic acid was performed and CS and conjugated CS-maleimide samples were freeze-dried and redispersed in deuterium oxide (D₂O). Conjugation reaction between the polysaccharide and the 3-maleimidopropionic acid group is described in **Supplementary Figure 1b**. Their ¹H-NMR spectra had been recorded from 0 to about 10 ppm using a proton NMR spectrometer, to confirm the successful conjugation. The typical CS spectrum was confirmed in **Figure 2b**, which presented a signal at $\delta \sim 4.9$, corresponding to H₇ anomeric proton, the ring proton chemical shift (H₂-H₆) as overlapping signals at $\delta \sim 3.6$ –4.0, which confirmed the presence of the

glucosamide moiety, while adjacent amino group ring proton (H_1) appeared at $\delta \sim 3.2$. The methyl group of the non-deacetylated part of the polysaccharide was expressed at $\delta \sim 2.1$ ($NHCOCH_3$), [39], and deacetylation degree $>85\%$ could also be confirmed basis of proton integrals. In **Figure 2a** there still appeared signals similar to that native chitosan signals at 2.1 ppm ($NHCOCH_3$), anomeric proton at 2.9 ppm (H_1) and glucosamide moiety at 3.8-4 ppm (H_2 to H_6) [40]. The presence of new peaks H_b and H_c at ~ 3.5 and 2.7 ppm, respectively, representative of typical protons of the maleimide moiety (**Figure 2c**), were in accordance with the chitosan chemical modification, proving the successful attachment of chitosan to maleimide propionic acid.

Fig. 2: 1H -NMR (400 MHz, D_2O) spectra and integration peaks of Chitosan-maleimide (a), Chitosan (b), 3-maleimidopropionic acid (c), and their respective chemical structures (right). All chemical shifts were expressed as δ in parts per million (ppm), using the chemical shift of the residual solvent resonances as references (D_2O : $\delta = 4.80$ ppm).

3.2. Preparation and physicochemical characterization of chitosan nanocapsules

3.2.1. Physicochemical characterization of nanocapsules

The combination of antigens with adjuvants into an optimal delivery system is a key for the development of a successful vaccine. In addition, aspects such as particle size or surface charge are crucial for the interaction and uptake of particles by immune cells [41]. The delivery carrier was selected based on the knowledge generated by our group and the protein antigen PsaA was associated on the surface of the nanocapsule to obtain a nanovaccine that mimics the pathogen structure and be able to generate a specific immune response.

CS NCs are made of a hydrophobic oily core surrounded by a hydrophilic coating. This core-corona structure uses lipids as core components allowing nanocapsules to interact with and penetrate through mucosal surfaces [42]. During the first step, we evaluated different ratios of surfactant:oil (data not shown) and the impact on the particle size and polydispersity of the resulting nanoemulsion (NE), and we selected nanometric size particles with narrow size distribution to form the oily core of the nanocapsules. The amount of Miglyol® was the main variable affecting the size of nanocapsules. Similar results were previously observed for other polymeric nanocapsule compositions such as those described by González-Aramundiz et al [21]. The use of non-ionic surfactants such as PEG-st 40 helps in preventing aggregation of particles during the formation of initial NE [43], whilst SGC was used as a co-surfactant that provides negative charge and enhanced stability to the oil core of the NCs [44]. As shown in **Figure 3**, chitosan molecules were anchored to the surface due to ionic interactions between polymer chains and negatively charged oily core, following an ionic interaction procedure, as previously described for chitosan coating colloidal systems [45], resulting in a core-coated nanostructure.

Fig. 3: Schematic representation of chitosan polymeric NCs developed in this work. Non-conjugated CS-antigen nanocapsules (left) and CS-antigen nanocapsules after thiol/maleimide conjugation (right). Design was created by Biorender® and ChemDraw® graphics programs.

NCs were prepared using the solvent displacement technique. As shown in **Table 1**, in all cases, they were monodisperse with a size range of 245-266 nm and the presence of a CS coating surrounding the oily core was corroborated by obtaining a positive surface charge of +30 mV, due to the amine groups in the CS structure. The NCs exhibited an increase in particle size and the surface charge decreased in the presence of antigen when compared with blank CS NCs. This increase in size could be considered as an indicator of a successful binding between the protein and the surface CS of the nanocapsules, and it could also be related to the presence of maleimide-groups as previously described for the development of thiolated-CS liposomes [46].

3.2.2. Association efficiency

Nanoparticle formulations prepared with thiol-maleimide coupling resulted in higher PsaA association efficiency than formulations without this covalent modification. Namely, the association efficiency of protein conjugated to the surface of NCs was approximately 3 times higher with PsaA-SATA compared to ones where the antigen was associated without polymer-protein conjugation reaction (86% versus 34%, approximately). Covalently conjugated NCs also preserved their particle integrity and physicochemical properties, so that they were expected to improve antigen delivery and activation of APCs, enhancing immunogenicity compared to non-conjugated NCs.

For uptake studies, the conjugation of CS-maleimide with Cy5 dye was first performed and nanocapsules were prepared using the same procedure previously described. The incorporation of the fluorophore did not alter the NCs original properties.

Formulation name	Size (nm)	Antigen:CS ratio	PDI	Z potential (mV)	Association Efficiency (%)
CS NCs	245 ± 20	-	< 0.1	31 ± 2	-
CS PsaA NCs	248 ± 21	1:5	< 0.1	29 ± 2	34 ± 9
CS-maleimide NCs	252 ± 23	-	< 0.1	31 ± 1	-
CS-maleimide PsaA NCs	266 ± 32	1:5	< 0.2	30 ± 1	86 ± 13
CS-maleimide Cy5 PsaA NCs	254 ± 16	1:5	< 0.1	29 ± 1	-

Table 1: Physicochemical characterization and association efficiency of antigen-conjugated and non-conjugated NCs. Particle size and polydispersity index were obtained by Dynamic Light Scattering. Data represent mean ± S.D., n=8. CS: Chitosan; NCs: Nanocapsules; PDI: Polydispersity index.

The NTA analysis (**Figure 4a**) of conjugated CS-maleimide PsaA NCs also confirmed previously reported DLS data showing a particle population of 268 ± 98.5 nm (red line), which corresponds to NCs. Analysis of the conjugated CS NCs in solution showed that the concentration/size distribution was less homogeneous than in case of lyophilized NCs, with more than one population of particles. Smaller NCs could be derived of NTA technique, where a higher dilution of CS-maleimide NCs (1:100) might had occasioned their rupture [47], while aggregation of primary NCs into large NCs could be consequence of the decrease of repulsive forces between the particle surfaces after chemical coating modification [48].

To assess the stability of conjugated CS-maleimide PsaA NCs after freeze drying process, formulations were lyophilized in the presence of trehalose 5%. After this procedure, the nanocapsules (purple line) were easily redispersed in ultrapure water and a slight change (< 50 nm) in the particle diameter was observed after NTA analysis. It was also observed that a lower concentration of nanocapsules tended to aggregate after lyophilization procedure compared with NCs in solution, which confirmed trehalose as an appropriate cryoprotectant to prevent nanoparticle aggregation. Production yield calculated by the difference obtained between the initial weight and the weight obtained after lyophilization was 67±21%. This freeze-dried chitosan nanocarrier could preserve their integrity upon storage without compromising its physicochemical properties, which is a very attractive feature in terms of developing a future thermostable vaccine formulation [49].

3.2.3. Morphological characterization

Different microscopic techniques were used to characterize the conjugated NCs. FESEM and STEM microscopy images revealed a homogeneous population of particles with round-shape structures, which confirm the spherical structure of the NCs. The average particle physical diameter was around 220 nm that corroborated the hydrodynamic NCs diameter values obtained by DLS, where slightly higher values were influenced by the interactions between the particles and the dispersion media (**Fig. 4b and 4c**) [50].

Figure 4: (a) Representative image of the particles size distribution by Nanoparticle Tracking Analysis of conjugated nanocapsules in solution (red line) and conjugated nanocapsules resuspended after lyophilization procedure (purple line), with the mean particle size $\pm$ standard deviation. Electron microscopy scanning (b) and transmission (c) images of conjugated Chitosan-maleimide PsaA NCs. Scale bars are indicated in each image. NCs: Nanocapsules.

3.2.4. Colloidal stability

Apart from obtaining adequate physicochemical properties, particle stability must be guaranteed for the developed nanosystems. The understanding of the colloidal stability and aggregation of the nanosystems in the biological media is important for their rational design. Moreover, the nanoparticle aggregation has shown to influence the *in vitro* cellular responses. Our studies showed that the size of the CS-maleimide PsaA NCs remained unchanged and no signs of aggregation were observed during a period of 24 h in both RPMI-1640 and SNF in suspension (**Figure 5a**), and after the freeze-drying process (**Figure 5b**), which suggest that the NCs were stable in the biological media and could be further proceeded for primary cell culture studies. However, there was a considerable increase in particle size when SNF medium was

supplemented with mucin type III. This increase in the particle size of the NCs can be attributed to the decrease in the Brownian motion of the particles due to higher viscosity of the medium in the presence of mucin, as well as structural properties of CS such as deacetylation degree or molecular weight [51]. This electrostatic interaction between the positively charged chitosan nanocapsules and the negatively charged mucin molecules yields protein-polysaccharide complexes [52]. According to Feng et al [53], dynamic viscosity could be calculated by DLS method, using water viscosity values (~0.89 cP at 25°C) and commercial polystyrene latex nanoparticles of known size (Magsphere Inc, USA) as reference. Values of ~1.21 cP for SNF media and ~1.41 cP for SNF media supplemented with mucin type III confirmed this increase in dynamic viscosity. Conjugated CS-maleimide PsaA NCs became larger and their brownian movement slower after incubation in SNF with mucin Type III, which might reflect the mucoadhesive properties of chitosan nanocapsules with mucin macromolecules [54]. Chitosan-mucin interaction by hydrogen and electrostatic bonding has been shown to be suitable for pharmaceutical and biomedical applications in the oral, nasal or ocular routes [55]. However, future in vivo experiments will be needed to confirm the mucoadhesive properties of CS-PsaA nanocapsules conjugated for nasal application. In addition, all formulations have maintained constant stability profiles in aqueous suspension at 4 °C for at least, 1 month (**Supplementary Figure 2**).

Fig. 5: Stability of conjugated CS-maleimide PsaA NCs in suspension (a) and lyophilized (b) conditions in water, cellular media (RPMI), simulated nasal fluid alone (SNF) and with mucine type III (SNF+ 0.1% Mucin). Bars represent size (nm), and dots represent derived count rate (kcps) (mean $\pm$ SD; n=3).

3.3. Release studies

The *in vitro* release behavior of PsaA from CS NCs was investigated using simulated nasal fluid (SNF pH = 6.5). The accumulative release study of non-conjugated CS-PsaA and conjugated CS-maleimide PsaA NCs was carried out after 24 h of nanocapsules incubation in SNF by analyzing the total amount of free protein in the media at each time point. Total loading was estimated considering the previously reported association efficiency rates achieved after physical (CS-PsaA NCs) and chemical (CS-maleimide PsaA NCs) conjugation. Values of protein released over time were obtained by extrapolation with a BSA protein concentration range in SNF. As observed in **Figure 6**, NCs presented an initial fast release during the first 3 hours of approximately the half of the PsaA antigen from the formulations to the medium. The release experiment indicated that the protein was gradually released from the nanocapsules in a time-dependent manner, and that this antigen release remained stable after 8 h. However, a sustained antigen release pattern

was observed after 3 hours in the CS-PsaA NCs, reaching values of 100% of the release at 24 h, contrary to the CS-maleimide NCs, where the release was slowed down, reaching values over 76% at 24 h. The relatively slower and sustained release in conjugated CS-maleimide PsaA formulation may be ascribed to the chemical conjugation which produced a stronger interaction between the antigen and the polysaccharide. As showed in similar studies in maleimide-bearing nanogels [56] and maleimide-functionalized PEGylated liposomes [57], carrier capacity to retain and release the drug was sustained for a prolonged time-period. Overall, both conjugated and free antigen release presented a biphasic profile with a faster initial release followed by a second phase with slow and delayed tendency release profile.

Fig. 6: *In vitro* cumulative release profile of PsaA antigen from chitosan PsaA and chitosan-maleimide PsaA NCs (mean $\pm$ S.D.; n=6). CS: Chitosan, NCs: Nanocapsules.

3.4. NCs toxicity on immune cells

To investigate the biocompatibility of both types of conjugated CS-maleimide nanocapsules (blank and antigen loaded), an *in vitro* MTS assay was performed to study the influence of the nanocapsules on the metabolic activity of macrophages and DCs. Since cationic nanoparticles generally have better electrostatic attractions with the negatively charged cell surface, their toxicity also might increase [58]. After 4 h (**Figure 7a**) and 24 h (**Figure 7b**) of CS-maleimide and CS-maleimide PsaA NCs incubation with macrophage Raw 264.7 cells, MTS assay showed a significant reduction in cell viability (mitochondrial reductase activity) at concentrations $> 200 \mu\text{g/mL}$. Membrane damage was more pronounced in blank CS-maleimide NCs, being practically total at values of $800 \mu\text{g/mL}$, while in the case of protein-conjugated nanocapsules it remained around 40% at higher doses. Similar effects on toxicity were observed after incubation of NCs

with iDCs for 24 h (**Figure 7c**), although in this case, toxicity was apparently higher in blank CS-maleimide NCs.

In a second instance, cell toxicity values were also verified *via* 7-AAD cytotoxicity assay test at 24 h. Considering that dendritic cells with compromised membranes were stained with 7-AAD, untreated cells were use as control for viable cells. The flow cytometry analyses performed (**Supplementary Figure 3**) showed a decrease in the number of viable cells as the CS-maleimide PsaA NCs concentration increased, reaching dendritic cell viability values of 65% at NCs concentrations of 900 µg/mL.

Fig. 7: Effect of CS-maleimide and CS-maleimide PsaA NCs on the viability of Raw 264.7 macrophages (a, b) and immature dendritic cells (c). MTS assay performed on Raw 264.7 cell lines incubated with either CS-maleimide (blue lines) or CS-maleimide PsaA (red lines) NCs using different concentrations of nanocapsules during (a) 4 h and (b) 24 h. (c) MTS assay performed on iDCs after incubating them with different concentrations of nanocapsules for 24 h. Results were normalized with the percentage of live iDCs incubated in culture media alone and dead iDCs incubated with Triton (5%, 1x) (mean $\pm$ S.D; n=4). CS: Chitosan; NCs: Nanocapsules.

3.5. Interaction of NCs with immune cells

Immune cells are able to capture antigens in many different ways, such as phagocytosis, macropinocytosis, or by expressing surface receptors causing endocytosis [59]. Once the antigen has been captured by the antigen presenting cells, it is processed and presented on the cell surface by Major Histocompatibility

Complex (MHC) [60]. To confirm whether our nanocarriers were able to interact with such immune cells, we examined the efficacy of CS-maleimide PsaA NCs on antigen uptake in primary cultures of human monocyte-derived iDCs and in the macrophage Raw 264.7 cell line. After incubating the Cy5-labelled NCs (both blank and antigen loaded), the presence of intracellular fluorescence due to the Cy5-labelled NCs confirmed the internalization of the nanocapsules by the DCs. The images obtained from the confocal scanning microscopy confirmed that the nanocapsules (top **Figure 8a**, red channel) were located within the dendritic cells (bottom, contrast microscope DiC channel) and intracellular distribution of CS-maleimide NCs could be seen throughout the different sections of dendritic cells (**also see online video resource in Supplementary material**). The physical appearance of DCs could also provide information about their maturation and activation status, for example, by the formation of their characteristic dendrites as seen in our case [25].

On the other hand, the interaction of macrophages with nanocapsules was analyzed by FESEM microscopy. The presence of protrusions on the surface of cells observed in FESEM images (**Figure 8c**) suggested a possible grouping of nanocapsules and recognition by macrophages.

Fig. 8: CLSM images of Cy5-labelled CS-maleimide PsaA NCs incubated with DCs (**a**) and FESEM images of Raw 264.7 cells alone (**b**) and after incubation with CS-maleimide PsaA NCs (**c**). Raw 264.7 cells (blue), and nanocapsules (purple, indicated with white arrows) in (b) and (c) were artificially colored to ease their visualization.

3.6. Human DCs phenotype analysis

To evaluate the effect that NCs may have on iDC phenotype, the expression of T lymphocyte costimulatory molecules CD83 and CD86 (indicators of pro-inflammatory activated phenotype in dendritic cells) were analyzed by flow cytometry. The co-stimulatory marker CD86 designates to be a marker primary DCs maturation expressed within first 24 h and interacts with the CD28 for the T cell activation, while the increased CD83 is observed in mature/activated DCs. Several studies have proven that antigen delivery to DCs upregulates expression of both CD83 and CD86 that are known to induce T cell receptor signalling and promote T cell activation [61]. In our study, both the blank and antigen loaded CS-maleimide NCs showed an increase in the expression of these maturation markers. The expression of CD83 and CD86 (**Figure 9a**) were 4 folds higher in the case of DCs primed with blank and antigen loaded CS-maleimide NCs when compared to iDCs (MFI=1). However, iDCs treated with PsaA alone displayed a 2 and 3-fold lower expression of CD83 and CD86 markers, respectively. Overall, the results suggest that either the blank or antigen loaded NCs can upregulate the co-stimulatory markers in comparison to the antigen alone.

3.7. NCs tolerogenic effect on immature DCs

Antigen-presenting cells such as iDCs play an important role in immune regulation, not only as inducers but also inhibitors of an immune response. 2,3-Indoleamine dioxygenase (IDO) is an enzyme involved in tryptophan catabolism, essential for the growth of microorganisms, which regulates T-cell tolerance [62]. IDO is a potent immune-suppressive enzyme in macrophages and dendritic cells whose physiological function is to catabolize the essential amino acid tryptophan into the stable metabolite, kynurenine which is known to induce the apoptosis of Th1 cells *in vitro* [63]. In addition, it is known that the IDO expression by the DCs can greatly impact peripheral tolerance and immune regulation. In this line, we performed a study to evaluate if the NCs formulations were inducing a tolerogenic phenotype on iDCs in terms of T-cell suppression and tolerance promotion (opposite to a pro-inflammatory response), by quantifying the IDO enzymatic activity after iDCs treatment with NCs. The results obtained from our study showed that the response in the DCs treated with either blank or antigen loaded CS-maleimide NCs signal was similar to one observed in iDCs (**Figure 9b**), indicating no tolerogenic effect of the NCs.

Fig. 9: (a) Quantification (%) of the expression of iDC maturation markers CD83 and CD86. Results are expressed as the ratio between the markers mean fluorescence intensity (MFI) in iDCs incubated with the different NCs and the MFI of iDC incubated in culture media alone (MFI=1). The dotted line corresponds to the signal from the iDCs. **(b)** Indoleamine 2,3-dioxygenase (IDO) activity in dendritic cell cultures. iDCs: immature dendritic cells; CS: Chitosan; NCs: Nanocapsules) (mean $\pm$ SEM; n=3 donors).

3.8. Activation CD4 and CD8 T cells by NCs

Stimulation of antigen specific CD4+ and CD8+ T lymphocytes is essential for efficient adaptive immunity. Therefore, we have assessed the ability of the DCs pulsed with antigen, blank CS-maleimide NCs or CS-maleimide PsaA NCs to activate T lymphocytes. The effect of conjugated NCs on the activation of T lymphocytes in an allogenic response was analyzed by flow cytometry. Blank CS-maleimide, CS-maleimide PsaA NCs (150 μ g/mL) and PsaA antigen alone were incubated with iDCs and then, the capability of this NC-incubated iDCs to activate allogenic lymphocytes was analyzed quantifying the expression levels of CD25 (for CD4 T lymphocytes) and CD28 (for CD8 T lymphocytes). As shown in **Figure 10**, mature DCs (mDCs) showed a greater capacity of activating both CD4 and CD8 T lymphocytes (black dots) than iDCs (open dots). On the other hand, preincubation of the iDCs with PsaA protein (green dots), blank CS-maleimide NCs (blue dots), and CS-maleimide PsaA NCs (red dots), induced similar percentages of activated CD4 and CD8 T lymphocytes when compared to iDCs.

In all cases, incubation of iDCs in the presence of NCs caused these APCs to have a similar capacity to stimulate CD4 and CD8 lymphocytes to iDCs, and were lower compared to mDCs. Also, levels of both activated CD4+CD25+ and CD8+CD28+ CD4 T cells were similar for loaded and non-loaded nanocapsules. However, no significant differences were found between treatments, probably due to donor variability. The similar values between both treatments on the effect of T cell activation could be a consequence of the composition of the nanocapsules, rather than the presence of the antigen on the surface. [64].

Fig. 10: Percentages (%) of activated T cells after iDCs incubation with formulations. Non-treated iDCs and LPS and IFN γ treated iDCs (mDCs) were used as controls (mean $\pm$ SEM; n=3 donors, 2-way ANOVA test). CS: Chitosan; iDCs: immature Dendritic Cells; mDCs: mature Dendritic Cells; NCs: Nanocapsules.

3.9. Cytokine secretion by PBMCs incubated with different NCs.

In the process of developing a powerful immune response, dendritic cells capture antigens thanks to pattern recognition receptors (PRRs), leading to phenotypic and maturation changes [65]. Depending on the alterations they undergo and the co-stimulation molecules they express, different responses will take place from the T cells (Th1, Th2 or Th17) [66]. The secretion of cytokines by Th1 CD4+ cells (such as IL-2, IFN γ , IL-10 or TNF α) is necessary in order to achieve protection against pneumococcal infection [2]. TNF α , secreted by either macrophages, monocytes or activated lymphocytes acts as an inflammatory marker and protects against the infectious pathogens. TNF α production can activate the T cells or can be a product of the activated T cells. In this procedure, we quantified by intracellular cytokine staining the production of TNF α by T lymphocytes (CD3+) and non-T cells (CD3-) after incubation with NCs. As can be seen in **Figure 11**, TNF α production was higher in the CD3- (monocytes) population, being around 5-8% compared to CD3+ (lymphocytes) population, where it did not exceed a 5% in any case. In both cases, a higher percentage of TNF α -positive cells was seen after incubated with the different NCs and the PsaA protein compared to iDCs, especially for the CD3+ T cells. Although no statistical differences were found in the different treatments due to the high variability among donors, the obtention of slightly higher cytokine secretion levels in CS-maleimide PsaA NCs could be a consequence of the presence of PsaA attached on their surface. These results were in agreement with previous studies evaluating the effect of cationic CS NCs in inducing inflammatory responses, where cytokine levels were remarkable in one out of three donors tested [67]. Likewise, when the PsaA protein was used, a clear increase in the percentage of TNF α -positive

cells was observed when compared to control cultures. This may indicate the external presence of the bacterial endotoxin lipopolysaccharide LPS, a potent activator of innate immunity.

Fig. 11: Percentages (%) of TNF α positive cells (CD3+ and CD3-) after treatment with CS-maleimide NCs, antigen loaded CS-maleimide PsaA NCs and PsaA antigen alone (mean $\pm$ SEM; n=3 donors, 2-way ANOVA test). CS: Chitosan; NCs: Nanocapsules.

4. Conclusion

In the design of targeted nanovaccines towards nasal immune cells, a specific nanocarrier design with mucoadhesive properties is crucial to achieve a higher residence time, avoiding mucociliary clearance and crossing nasal epithelium. In this work, combination of the antigenic protein membrane PsaA together with the high capacity of drug association of chitosan-maleimide modified nanocapsules has been positioned as an interesting perspective for *Streptococcus pneumoniae* vaccination. Results supported the versatility of non-capsular pneumococcal antigens as vaccine candidates for an efficient mucosal administration. Developed nanocapsules presented a high effective payload of antigen after covalent thiol linkage, mucoadhesiveness together with high physical and biological stability. The nanocapsules capacity to interact with immune cells was conserved and enhanced compared to the free antigen, suggesting them a “green light” future serotype-independent pneumococcal vaccine candidate. To further assess the ability of these nanocapsules as vaccine delivery systems, the evaluation of antigen-specific responses and cytokine profiles is currently under way.

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Conflicts of interest/Competing interests

Authors declare that they have no conflict of interest.

Ethics approval

All procedures followed were in accordance with the ethical standards of the responsible institutional and national committees on human experimentation and with the Declaration of Helsinki. Informed consent was obtained from all patients for being included in the study.

Consent to participate

All institutional and national guidelines for the obtention and use of human blood were followed. Blood was drawn with the informed consent of all subjects and appropriate permission was obtained from the Institutional Ethics Committee (Comité Ético de Investigación Clínica de Galicia, CEIC). No animal or human studies were carried out by the authors for this article.

Consent for publication

Not applicable

Availability of data and material

Not applicable

Code availability

Not applicable

Authors' contributions

Sandra Robla was in charge of the overall experimental execution, data analysis, writing and figure preparation. Maruthi Prasanna contributed to the experimental execution and writing. Cyrille Grandjean was involved in the synthesis and characterization of the PsaA antigen, reviewing and editing the manuscript. R Varela-Calvino was involved in the design and supervision of the in vitro cell experiments, reviewing and editing the manuscript. Noemi Csaba was involved in writing, reviewing, editing, supervision, project design and administration.

Supplementary material

Supplementary Figure 1: (a) Chemical conjugation scheme between PsaA and N-succinimidyl S-acetylthioacetate (SATA) group and (b) Chitosan and 3-maleimidopropionic acid group. Reaction synthesis yield after purification and lyophilization procedure was ~ 30% and 50%, respectively.

Supplementary Figure 2: Stability of conjugated CS-maleimide PsaA NCs after 1 month under storage

conditions (dark and 4°C). Bars represent size (nm), and dots represent derived count rate (kcps) (mean± SD; n=6). CS: Chitosan; NCs: Nanocapsules.

Supplementary Figure 3: 7-AAD cytotoxicity study in function of the concentration of the conjugated CS-maleimide PsaA NCs. (mean± SD; n=3). CS: Chitosan; NCs: Nanocapsules.

Online resource 1: Intracellular distribution of Cy5-labelled CS-maleimide PsaA NCs throughout the different sections of DCs after confocal microscopy.

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