Progress in mucosal immunisation for protection against pneumococcal pneumonia

Viviane Maimoni Gonçalves*[1], Kan Kaneko[2], Carla Solórzano[3], Ronan MacLoughlin[4], Imran Saleem[5], Eliane Namie Miyaji[6]

*Corresponding author

1. Laboratory of Vaccine Development, Instituto Butantan

Av Vital Brasil, 1500, Sao Paulo, SP, Brazil

phone: +55 11 26279819

e-mail: viviane.goncalves@butantan.gov.br

2. School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University

James Parsons Building, 3 Byrom Street, Liverpool, L3 3AF, UK

phone: +44 (0)151 231 2456

e-mail: K.Kaneko@ljmu.ac.uk

3. Department of Clinical Sciences, Liverpool School of Tropical Medicine

Pembroke Place Liverpool L3 5QA, UK

phone: +44 (0) 151 705 3788

e-mail: Carla.SolorzanoGonzalez@lstmed.ac.uk

4. Aerogen Ltd.

IDA Business Park, Dangan, Galway, Ireland

phone:+353 91 540400

e-mail: RMacLoughlin@aerogen.com

5. School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University

James Parsons Building, 3 Byrom Street, Liverpool, L3 3AF, UK

phone: +44 (0)151 231 2265

e-mail: I.Saleem@ljmu.ac.uk

6. Laboratory of Bacteriology 2, Instituto Butantan

Av Vital Brasil, 1500, Sao Paulo, SP, Brazil

phone: +55 11 26279827

e-mail: eliane.miyaji@butantan.gov.br

Abstract

Introduction: Lower respiratory tract infections are the fourth cause of death worldwide and pneumococcus is the leading cause of pneumonia. Nonetheless, existing pneumococcal vaccines are less effective against pneumonia than invasive diseases and serotype replacement is a major concern. Protein antigens could induce serotype-independent protection, and mucosal immunisation could offer local and systemic immune responses and induce protection against pneumococcal colonisation and lung infection.

Areas covered: Immunity induced in the experimental human pneumococcal carriage model, approaches to address the physiological barriers to mucosal immunisation and improve delivery of the vaccine antigens, different strategies already tested for pneumococcal mucosal vaccination, including live recombinant bacteria, nanoparticles, bacterium-like particles and nanogels as well as, nasal, pulmonary, sublingual and oral routes of vaccination.

Expert commentary: The most promising delivery systems are based on nanoparticles, bacterial-like particles or nanogels, which possess greater immunogenicity than the antigen alone and are considered safer than approaches based on living cells or toxoids. These particles can protect the antigen from degradation, eliminating the refrigeration need during storage and allowing the

manufacture of dry powder formulations. They can also increase antigen uptake, control release of antigen and trigger innate immune responses.

Keywords: *Streptococcus pneumoniae*, pneumococcal surface protein A, serotype-independent pneumococcal vaccines, experimental human pneumococcal carriage, nanoparticles, bacterial-like particles, nanogels, live recombinant bacteria, outer membrane vesicles

1. *Streptococcus pneumoniae* is the leading cause of lower respiratory tract infections

Streptococcus pneumoniae, also known as pneumococcus, is a Gram-positive bacterium that colonises asymptomatically the nasopharynx of humans [1], but can also invade other niches and cause non-invasive diseases such as acute otitis media, sinusitis and non-bacteraemic community acquired pneumonia (CAP), and when pneumococcus invades normally sterile sites, it gives rise to the life-threating invasive pneumococcal diseases (IPD), such as bacteraemic pneumonia, empyema, meningitis and sepsis [2].

Studies performed by the Global Health Metrics point out lower respiratory tract infections (LRI) as the fourth cause of death worldwide and pneumococcus is estimated to be the leading cause of LRI mortality in children under 5-years old [3]. Nonetheless, a decrease in deaths caused by pneumococcal pneumonia was observed in children younger than 5 years, while an increase occurred among adults over 70 years in the period 1990–2017 [3]. These two facts were related to the introduction of pneumococcal conjugate vaccines in universal children vaccination programs of several countries and to the serotype replacement phenomenon that is particularly important in adult populations [4,5].

2. Existing pneumococcal vaccines

Capsular polysaccharide is the main *S. pneumoniae* virulence factor and the antigen for all currently available pneumococcal vaccines. Capsular polysaccharides are also the basis for classification of pneumococcus in more than 95 serotypes, each serotype corresponding to a chemical and immunologically distinct polysaccharide [6].

There are two types of vaccines, both administered via the parenteral route: 23-valent pneumococcal polysaccharide vaccine (PPV-23), and pneumococcal conjugate vaccines, PCV10 and PCV13, the valence corresponding to the number of polysaccharides from different serotypes included into vaccine formulation. As a T-cell independent antigen, polysaccharides can induce serotype-specific antibody, but no immunological memory [7]. Therefore, pneumococcal polysaccharides from prevalent serotypes were chemically conjugated to carrier proteins in order to be protective in children under 2-years-old [7,8]. Today, the World Health Organization (WHO) recommends the inclusion of PCVs in immunisation programmes worldwide for children under 5-years-old [9]. PPV23 is recommended for at risk population >2 years and adults >65 years of age and PCV13 has been adopted in some developed countries for immunisation of adults > 50 years old [8].

An important effect of PCV introduction in childhood vaccination programmes is the herd protection observed in non-vaccinated population [10]. Several studies have demonstrated the herd effect, resulting in the reduction of vaccine type diseases [11-14]. Nevertheless, there is still a high level of global mortality and morbidity caused by IPD, owing to the limited serotype coverage of PCVs, which results in replacement of serotypes included in vaccines by non-vaccine serotypes [14-16]. It has been shown that PCV elicits antibody responses against the capsule of the serotypes included in the vaccine, reducing carriage prevalence and density by those serotypes [17-21]. Therefore, PCV alters the microbiota in the nasopharynx leaving a vacant niche that could be occupied by non-vaccine serotypes and other respiratory pathogens such as *Staphylococcus aureus* and nontypeable *Haemophilus influenzae* (NTHi), potentially increasing respiratory infections. Following the implementation of PCVs, studies in

different settings have reported the increased incidence in carriage and IPD caused by non-vaccine serotypes, indicating serotype replacement [22-32]. The serotype replacement mitigates the benefits of vaccination and has compelled pharmaceutical companies to develop higher-valency PCVs [33-36]. In addition, several initiatives are being directed to the development serotype-independent vaccines [37-41], including the addition of pneumococcal proteins to PCV formulations [42-44].

As mentioned, other pathogenic bacteria could replace the niche previously occupied by vaccine type serotypes. Randomized controlled trials and observational studies have reported an inverse association between *S. pneumoniae* with both *H. influenzae* (mainly NTHi) and *S. aureus* after PCV vaccination [45-49]. Both *S. aureus* and NTHi have been found significantly increased in PCV-vaccinees, specially causing episodes of acute otitis media [49-52]. Other studies conducted in South Africa [53], the US [54] and Greenland [55] have reported declined of *H. influenzae* and *S. aureus* carriage in PCV vaccinees.

Novel vaccine strategies could solve issues regarding serotype replacement and could potentially increase protection against pneumococcal diseases; however, there will be a need for disease surveillance to monitor their effect over respiratory infections caused by other pathogenic bacteria.

3. Effectiveness of current pneumococcal vaccines against pneumonia

Pneumonia can be caused by viruses, bacteria, or fungi [56]. Recent data have demonstrated that the aetiology of pneumonia is not yet well established and studies are strongly influenced by factors as high sensitivity and low specificity of case definition, prior antibiotic treatment, access to healthcare, specimens collected from sites distant from the lungs, presence of multiple potential pathogens in the specimens collected, underrepresentation of fatal cases and bias related to the identification methods [57]. Nonetheless, *S. pneumoniae* is recognized as the main cause of bacterial pneumonia, followed by *H. influenzae*, while respiratory syncytial

virus is the most common viral cause of pneumonia [3]. Also, relationship between previous virus infection and predisposition to bacterial infection has been reported [58]. Vaccine probe studies have contributed to point out pneumococcus and *H. influenzae* as important causes of pneumonia [57].

Despite the difficulties to attribute an unequivocal aetiology to pneumonia, association between the introduction of pneumococcal vaccines and the reduction of mortality among children younger than 5 years of age was observed, but an increase among older adults was also reported [3]. Although case definition and methodologies vary, recent studies demonstrated that PCVs provide protection against CAP in children, especially for severe cases, after vaccine implementation, and that most non-PCV13 serotypes presented lower invasiveness than vaccine serotypes [59-63].

In adults, different meta-analysis studies could not consistently demonstrate PPV23 effectiveness [64-67], which is in accordance with the lack of induction of immunological memory by polysaccharide antigens [7]. However, the vaccine effectiveness of PPV23 for older adults remains controversial, as shown by a recent review about this subject [5]. Following the lack of unequivocal effectiveness of PPV23 against pneumonia, the immunisation of adults with PCV13 was investigated in double-blind placebo-controlled randomised clinical trial conducted in adults aged ≥65 years (CAPiTA study) in the Netherlands [68]. Post-hoc analyses showed lower incidence of CAP and a modest reduction in the hospitalisation rate and stay time among the vaccinated individuals [69,70], and similar results were observed in US [71,72], Italy [73], and Spain [74,75].

It is important to emphasize, however, that PCVs were less effective at preventing pneumococcal CAP compared to IPD and the effectiveness was obviously higher for vaccine type diseases than for non-vaccine type [68,76], indicating the potential for serotype replacement. Also, the high cost of PCVs hampers their introduction in low and middle-income countries [76]. Therefore, there is a need for

novel approaches to fight pneumococcal pneumonia and mucosal immunisation is one of the most promising strategies to address this problem.

4. Formulation approaches for mucosal immunisation against *S. pneumoniae*

There are several important considerations for vaccination through the nasal and pulmonary routes, as they exhibit unique physiological properties. Numerous approaches have been reported for addressing the physiological challenges and improving delivery of the vaccine antigens (Figure 1).

4.1 Mucus layer

The mucus layer is a major barrier encountered in both nasal and pulmonary delivery, as it forms a physical obstacle between the immune cells and the formulation [77]. In addition to functioning as a barrier, the mucociliary action of the surface results in clearance. For these reasons, formulations with mucoadhesive and mucopenetrative properties have been of interest [78]. Hydrophilic polymers such as carbopol, sodium alginate, pullulan, exhibit mucoadhesive effects through the formation of hydrogen bonds with the mucus [78,79]. Chitosan is another popular polymer that can interact with the mucus through ionic interactions, and also possesses additional immunogenic properties through opening of intercellular tight junctions and the activation of STING-cGAS pathway [77,80]. The incorporation of such polymers has been shown to increase the retention time of the formulation at the mucosal site and postulated to promote greater local immune responses. In the peripheries of the lungs, the epithelium is covered by a surfactant layer which is composed predominantly of phospholipids [81]. Preparing formulations from these phospholipids is postulated to be beneficial for reducing the risk of toxicity, which is a recognised concern especially for particulate formulations [81].

4.2 Antigen delivery and uptake

Despite the acknowledgement that nasal and pulmonary immunisation can result in mucosal and systemic immune responses, the underlying physiology and mechanisms of how the formulations interact with the immune system are still not well understood [78]. The uncertainty of targets means that it is difficult to develop formulations which can target specific tissue or cell types.

However, there are numerous non-specific targeting approaches that are known to improve immunostimulation, based on aspects such as improving uptake by antigen presenting cells and prolonged release of the antigen and/or adjuvant. It has been widely established that nanoparticle (NP) formulations exhibit these properties [82], and thus are of particular interest for mucosal vaccine formulations. Another consideration especially for particulate formulations is the ubiquitous presence of alveolar macrophages, which exhibit high activity and clear foreign material from the airways [81]. The clearance and the potential immune response by the alveolar macrophages should be considered for potential formulations.

4.3 Immune cell stimulation

Serotype independent antigens, such as pneumococcal surface protein A (PspA), are generally protein or subunit based antigens, and are normally safer compared to the whole cell or viral vectors [83]. However, this low immunogenicity presents a challenge for formulating vaccines, and thus an adjuvant is generally required for inducing sufficient immune responses. Molecular adjuvants, such as cytokines, toll-like receptors agonists and nucleic acids, can be incorporated into the formulation with the antigen to improve immunogenicity [82]. Adjuvants can also be delivery vehicles, such as nanoparticles, emulsions and hydrogels [83]. Such formulations can improve the antigen exposure to the immune cells and also improve uptake by the antigen presenting cells. Although there are currently no approved intranasal or pulmonary protein vaccines, several preclinical formulations using serotype-independent antigens against *S. pneumoniae* have been explored.

5. Human immunity induced in the lungs following nasal immunisation with live pneumococcus

The pneumococcus frequently colonises the human nasopharynx and this exposure elicits both humoral and cellular responses that have an immunising effect in humans [84,85]. An effective control of colonisation and an active alveolar macrophage-mediated immune response in the lung are thought to be essential for protection against pneumococcal pneumonia [86]. In humans, immunoglobulin deficiencies [87], co-infections [88] and polymorphisms in the IL-17A gene [89] increase the incidence of lung infection. These data suggest an important role for antibodies and Th17 $CD4^+$ T cells in protection against pneumonia.

Experimental human colonisation studies have allowed to characterise the effects of colonisation with live *S. pneumoniae* bacteria in lung immunity. In the experimental human pneumococcal carriage (EHPC) model, healthy volunteers are intranasally inoculated with a pneumococcal serotype 6B strain leading to successful colonisation in approximately half of subjects [85,90]. In these studies, bronchoalveolar lavage fluid (BALF) is obtained from colonised and non-colonised volunteers to characterise lung mucosa immune responses. Colonisation induced by experimental inoculation increases the percentage of IL-$17A^+$/TNF^+ $CD4^+$ memory T cell in BALF when comparing to non-colonised individuals after *ex vivo* stimulation with serotype 6B [84]. Additionally, production of IL-17A from lung cells stimulated with 6B pneumococcus is high in both colonised and non-colonised volunteers and this cytokine plays an important role improving alveolar macrophage-mediated *S. pneumoniae* killing [84]. EHPC has also demonstrated that intranasal inoculation with live bacteria in absence of colonisation increases antibody levels against pneumococcal proteins such as PspA [91].

More recent data using EHPC has shown that experimental colonisation, through micro-aspiration, boosts the innate lung immunity. It increases the

opsonophagocytic capacity of alveolar macrophages against pneumococcus and the expression of pro-inflammatory cytokines such as IFN-γ and TNF-α [92]. This human data emphasises the benefits that nasal inoculation of live bacteria has upon lung immunity and as an attractive immunisation approach for pneumonia prevention. The induction of pneumococcal specific Th1/Th17 cellular and humoral responses and the non-specific boosting of innate lung immunity could potentially play a pivotal role in protection against pneumococcal pneumonia in humans.

6. Immunity induced in the lungs following nasal immunisation with pneumococcal protein-based vaccines

Human and murine models have shown that mucosal exposure to pneumococcus elicits both mucosal and systemic humoral and cellular responses. Therefore, mucosal vaccination represents an attractive approach for immunisation as it mimics the natural route of pneumococcus infection. Intranasal immunisation with PspA has been largely studied showing promising results in protection against pneumococcal lung infection in animal models [93-98]. Early studies (Table 1) have shown that intranasal immunisation with PspA co-administered with a mucosal adjuvant such as cholera toxin subunit B (CTB) or non-toxic cholera toxin is protective against pneumococcal challenge models of pneumonia and induces serum IgA and IgG to PspA [93,94]. Current studies are focused in developing non-toxin-based nasal vaccine delivery systems to enhance the efficacy of PspA and other protein-based vaccine candidates against pulmonary infections [84].

Other than describing different protein antigens used for mucosal immunisation, this review will focus on the different strategies already tested for mucosal vaccination, including live recombinant bacteria (Table 2), nanoparticles, bacterium-like particles and nanogels (Table 3). The protein antigens most commonly tested in mucosal immunisation are PspA and pneumococcal surface antigen A (PsaA) and they will be addressed here. Literature on mucosal immunisation includes not only nasal and

pulmonary routes of vaccination, but also sublingual and oral routes. Finally, the use of non-protein antigens for mucosal immunisation against pneumococcal infections will be discussed. Since there is only a limited number of papers regarding mucosal formulations specifically for pneumococcus, we included everything there was in the databases about it, without limits on when the paper was written.

7. Live recombinant *Salmonella* and Outer Membrane Vesicles for oral and nasal immunisation

A live-attenuated strain of *Salmonella enterica* serovar Typhimurium expressing PspA was developed for use in oral immunisation of mice (Table 2). Oral immunisation induced anti-PspA IgG in serum and vaginal secretion, protecting mice against intraperitoneal lethal challenge with serotype 3 pneumococcal strain WU2 [99]. *S.* Typhimurium expressing PspA was also tested for protection against secondary pneumococcal pneumonia in mice. In this model, mice were intratracheally challenged with strain WU2 one week after intratracheal challenge with influenza virus PR8. A single oral dose protected mice from secondary pneumonia, resulting in attenuated pulmonary inflammation, reduction in bacterial loads in the lungs and increased survival. The immunisation induced anti-PspA IgG antibodies in serum and also IgA antibodies in serum and BALF [100].

A Phase I dose escalation trial was conducted to evaluate the safety and immunogenicity of three recombinant attenuated *Salmonella enterica* serovar Typhi vaccine vectors expressing PspA (Table 2). These strains were attenuated *in vivo* due to the absence of arabinose and expression of the antigen is delayed until after invasion of the host intestinal tissues. The group of volunteers receiving the highest dose (10^{10}) through the oral route did not show increase in anti-PspA titers compared to baseline. The authors discuss that immunogenicity may have been limited due to pre-existing cross-reactive antibodies to *S.* Typhi [101].

Outer membrane vesicles (OMVs) from recombinant *Salmonella* have also been tested for mucosal vaccination against pneumococcal infections (Table 3). OMVs are formed by blebbing of the outer membrane of Gram-negative bacteria and contain periplasmic components. OMVs were purified from *S.* Typhimurium expressing PspA in the periplasm. Intranasal immunisation of mice with the OMVs elicited significant anti-PspA IgG responses in the serum and weak mucosal IgA. Immunised mice showed complete protection against a low dose pneumococcal intraperitoneal challenge with serotype 3 strain WU2 and partial protection against high dose challenge [102]. OMVs from *S.* Typhimurium displaying fragments of PspA on the surface were used also to immunise mice intranasally. Protection against pneumococcal nasal colonisation with serotype 4 strain TIGR4 was observed in immunised mice, which was correlated with local production of antigen-specific IL-17A [103].

8. Live recombinant lactic acid bacteria and bacterium-like particles for nasal and sublingual immunisation

Recombinant lactic acid bacteria (LAB) expressing pneumococcal antigens were also tested as vaccine (Table 2). LAB are microorganisms present in the gastrointestinal mucosa of healthy individuals that are widely used in dietary products and are generally recognized as safe. *Lactococcus lactis*, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus helveticus* expressing PsaA directed to the cell wall were used in intranasal immunisation experiments in mice. Higher levels of specific serum IgG and mucosal IgA were detected in mice immunised with *L. plantarum* and *L. helveticus*. Vaccination with recombinant lactobacilli but not with recombinant *L. lactis* led to a decrease in *S. pneumoniae* recovery from nasal mucosa upon a colonization challenge with serotype 6B strain 0603. These results show that some *Lactobacillus* strains have intrinsic properties that make them suitable candidates for mucosal vaccination [104].

L. lactis expressing PspA intracellularly was used for intranasal immunisation of mice and induced anti-PspA IgG in serum (Table 2). Antibodies showed a balanced IgG1/IgG2a ratio, in contrast to immunisation with recombinant protein adjuvanted with alum inoculated subcutaneously. Immunisation with recombinant LAB afforded protection against lethal intraperitoneal and respiratory challenges against serotype 4 strain TIGR4. Protection against respiratory challenge was higher than for vaccination with recombinant protein. Furthermore, animals immunised with live bacteria showed better protection than those inoculated with inactivated bacteria. Protection elicited by the recombinant LAB was associated with an IgG response with a Th1 profile [105]. Intranasal immunisation of mice with *L. casei* expressing PspA led to the induction of specific serum IgG but not of mucosal IgA. Partial protection against intraperitoneal challenge with serotype 3 strain A66.1 was observed [106].

Bacterium-like particles (BLPs) are based on acid-treated *L. lactis*, consisting of a peptidoglycan cell wall surrounding a single membrane [107]. The acid treatment degrades components inside the cell and within the cell wall, leaving a particle resembling a bacterial cell. They have similar shape and size to bacteria and can act as vaccine adjuvants to stimulate the immune system and enhance mucosal immunity (Table 3). PspA was incorporated onto the surface of these BLPs and intranasal immunisation of mice resulted not only in high levels of serum IgG antibodies, but also high levels of mucosal secretory IgA (SIgA) antibodies in the respiratory tract [108]. Moreover, mice were protected against fatal intranasal challenge with homologous and heterologous pneumococcal strains and bacterial load also decreased in the lungs, showing serotype independent protection [109]. Similar results were obtained when loading PspA and PsaA into BLPs [98]. The adjuvant effects of BLPs are well characterised, involving the activation of DCs through the TLR [107].

9. Nanoparticles and nanogels

9.1 Nanoparticles

Nanoparticle (NP) formulations such as polymeric NPs and liposomes have been shown to possess greater immunogenicity compared to the antigen alone [80]. This is thought to be due to mechanisms such as increased antigen uptake, controlled release of antigen and triggering the innate immune response [80]. There are several mucosal NP formulations which have been reported to exhibit protective effects against *S. pneumoniae* (Table 3). The mucosal-delivered formulations loaded with PspA have shown similar effects on the lung mucosa, inducing the production of PspA-specific antibodies in serum and BALF, reducing pneumococcal load and protecting against pneumococcal lethal challenge in murine models [96,110].

A NP formulation made from poly(glycerol adipate-co-ω-pentadecalactone) (PGA-co-PDL), with surface adsorbed PspA, resulted in increased antigen-specific IgG antibodies in the serum [96]. Challenge with the lethal pneumococcal serotype 3 strain ATCC6303 showed that the group immunised with the NP formulation exhibited lower bacterial load in the lungs, as well as increased survival (Table 3). The NP administered through the nose targeting the lungs also contributed to greater PspA specific IgG antibody titers in BALF than subcutaneous administration of PspA, which could have contributed to earlier control of the infection. These NPs were also formulated as larger spray-dried microparticles made from L-leucine, which confers several benefits, such as the possibility for direct inhalation into the lungs, as the particle size can be modified for optimal lung inhalation [111]. The dry powder form also improves stability, as no refrigeration is required during storage. Moreover, the formulation can be resuspended in an aqueous solvent for inhalation by nebulisation. These properties improve the practicality of the mucosal administered formulation.

Another reported particulate formulation is the polysorbitol transporter (PST), which is made up of sorbitol diacrylate and low-molecular-weight polyethylenimine [110]. The polymer possesses osmotic properties that cause the cells to increase particle uptake through the caveolae-mediated pathway. Intranasal immunisation with PST formulation incorporating PspA induces DCs activation, associated with a Th2 or

follicular helper T cell responses (Table 3). This resulted in generation of long-term memory antibody-producing cells and long-term protection against *S. pneumoniae* WU2. The mechanism is postulated to involve the induction of PPAR-γ expression in antigen presenting cells, which can control the Th2, or anti-inflammatory immune responses, as well as the generation of memory B cells [110].

Chitosan NPs incorporating DNA encoding PsaA were also shown to protect mice against nasopharyngeal colonisation when administered intranasally [112]. The positive charge of the chitosan allows for complexation with the negatively charged DNA, and has been shown to be an effective mucosal gene carrier for other applications. Chitosan-DNA nanoparticles had an average size of 392 nm. Mice immunised intranasally with the nanoparticles showed higher anti-PsaA IgG both in serum and nasal washes when compared to animals inoculated with the naked DNA (Table 3). IgG response was characterized by a balanced IgG1/IgG2a ratio and by the secretion of IL-17A and IFN-γ by spleen cells [113].

Chitosan nanoparticles containing encapsulated PsaA protein was also evaluated for nasal immunisation of mice (Table 3). Particles had an average size of 691 nm and immunisation elicited higher IgG levels in the serum and higher IgA levels in nasal wash, BALF and middle ear lavage when compared to the naked protein. The immune response was characterized by the secretion of IL-17A, IL-4 and IFN-γ by spleen cells. Moreover, clearance of a serotype 14 strain in the middle ear and protection against intraperitoneal challenge with serotype 3 and serotype 14 strains were observed [114].

Although there are few applications of NPs against *S. pneumoniae*, these reported studies, in addition to numerous other studies investigating NP use against other pathogens, suggest that NP vaccines are a viable approach for mucosal immunisation [78].

9.2 Nanogel

In addition to NPs, a cationic cholesteryl pullulan (cCHP) nanogel that incorporates PspA has been investigated as a nasal vaccine formulation [115]. These gels are formed through hydrophobic interactions and can prolong the release of incorporated proteins, to improve immunogenicity.

After immunisation with the nanogel, mice exhibited high levels of serum PspA-specific IgG, and nasal and bronchial IgA responses, as well as systemic and mucosal Th17 responses (Table 3). Immunisation with this formulation protected mice against lethal challenge with *S. pneumoniae* Xen10 (derived from A66.1), which carries homologous PspA, as well as against serotype 3 strain 3JYP2670, which expresses heterologous PspA, and reduced colonisation and invasion in the upper and lower respiratory tracts by Xen10 [115].

A similar formulation with a cationic group-modified cCHP nanogel was tested to improve efficacy in macaques and shown to be effectively delivered to the nasal mucosa, being retained in the nasal tissues for up to 6 hours (Table 3). Serum IgG and mucosal IgA responses were observed. In addition, no PspA was found in the olfactory bulbs or the central nervous system, suggesting that the nanogel is safe and does not cause unnecessary trafficking of the antigen to vulnerable areas which may lead to unwanted consequences. Moreover, serum from immunised macaques passively protected mice from an intravenous lethal challenge with strains Xen10 and 3JYP2670. Cellular immune response was characterized by the secretion of Th2 and Th17 cytokines by $CD4^+$T cells [116].

10. Mucosal immunisation with non-protein antigen: phosphorylcholine, cell wall polysaccharide and capsular polysaccharide

Non-protein antigens have been tested as mucosal vaccines against pneumococcal infections in the first attempts for mucosal immunisation (Table 1). Intranasal immunisation with phosphorylcholine conjugated to porcine thyroglobulin was shown to induce antibodies in serum and BALF and to protect mice against a

lethal challenge with a serotype 3 strain [117]. Inactivated pneumococcal whole cell and cell wall polysaccharide given intranasally to mice with cholera toxin or CTB as adjuvant were also shown to increase resistance to nasopharyngeal colonisation by serotype 6B strain 0603 [118,119]. Anti-cell wall polysaccharide serum IgG antibodies were predominantly directed against the phosphorylcholine component. Protection was shown to be independent of antibodies, but dependent on $CD4^+$T cells and IL-17A. The immunisation strategy also protected in a model of fatal aspiration pneumonia by serotype 3 strain WU2 [119].

Capsular polysaccharides have also been used for mucosal immunisation. Serotype 1 and 3-tetanus toxoid conjugate vaccines were used for intranasal immunisation of mice with the adjuvant RhinoVax (Table 1). The adjuvant formulation is based on caprylic-capric glycerides dissolved in polysorbate 20 and water. Serum IgG and IgA response was observed for serotype 1 and immunised mice showed lower bacterial loads after 24 hours after challenge with serotype 1 strain 6301. Lower serum IgG levels were observed for serotype 3. Nevertheless, animals were protected against an intranasal lethal challenge with a serotype 3 strain ATCC6303 [120]. Nasal administration of these conjugate vaccines with LT-K63 and LT-R72 as adjuvants also induced protection against pneumococcal challenge [121].

Serotype 3 and serotype 14-CRM_{197} conjugate vaccines were tested in intranasal immunisation using IL-12 as adjuvant (Table 1). Specific IgG and IgM antibodies were detected in the serum of mice immunised with the serotype 3 conjugate vaccine, whereas specific IgG, IgM and IgA antibodies were detected in the BALF. The use of IL-12 as adjuvant led to the increase in IgG2a antibodies when compared to animals immunised without adjuvant. When comparing immunisation with the conjugate vaccine plus IL-12, intramuscular vaccination led to higher survival after intraperitoneal challenge with serotype 3 strain A66.1, whereas intranasal immunisation led to higher protection against nasopharyngeal colonisation challenge with a serotype 14 strain. Protection against colonisation was shown to be dependent on IgA [122].

Finally, lung immunisation with PPV23 has also been reported in humans (Table 1). Alveolar and bronchial immunisation with PPS23 using a nebulizer was compared to intramuscular administration in healthy human volunteers and also in patients with chronic obstructive pulmonary disease (COPD). PPV23 was shown to be safe to be administered by controlled inhalation and to induce serum antibody responses, albeit at lower levels when compared to intramuscular immunisation [123,124]. Another study reported negative results for pulmonary immunisation with PPV23 using a jet nebulizer. Bronchoalveolar and serum antibody responses were compared in volunteers immunised after injected or inhaled PPV23. Increase in IgG and IgA in both serum and BALF of individuals was observed in inoculated via the intramuscular route, but not via the pulmonary route with alveolar deposition [125].

Expert opinion

Conjugate vaccines are highly effective against IPD, reduce nasopharyngeal colonisation and induce herd immunity. However, the protection is limited to the serotypes included in the formulations, leading to serotype replacement in the population. Moreover, the effectiveness of all current vaccines is much greater for IPD than community-acquired pneumonia, even when only vaccine type pneumonia is considered. Therefore, serotype-independent vaccines could deal with the serotype replacement and mucosal immunisation could fill the gap for pneumococcal pneumonia protection.

Several antigens are promising candidates for providing serotype-independent protection, and PspA is the most studied and has been applied to all strategies for mucosal immunisation: administered with mucosal adjuvants as CTB, expressed in live recombinant bacteria, expressed in *Salmonella* OMV, attached to BLP, and formulated in nanogels and nanoparticles (Tables 1-3). Hence, it is clear that PspA should be included in any new pneumococcal vaccine formulation. However, PspA is classified in 3 families and 6 clades according to the amino acid sequence. Immunological cross-

reactivity occurs inside each PspA family and is higher among variants of the same clade. Consequently, new research should answer how many PspA variants from different clades should be included in a new vaccine, and recent works addressed this problem by generating hybrid molecules or formulating vaccines with a mix of different PspA variants. Furthermore, there is a question whether only PspA from different families will be sufficient to offer full protection and avoid a phenomenon similar to the serotype replacement. Thus, other pneumococcal antigens should be considered, for example, the immunisation with PsaA offered protection against nasopharyngeal colonisation, which should be useful for nasal vaccines, and antibodies against the pneumococcal toxin pneumolysin (Ply) could prevent tissue damage and local inflammation, so detoxified Ply mutants could enhance the protection offered by PspA, especially for lung immunisation.

The data presented here support the thought that intranasal immunisation with protein-based vaccines could provide a better protection against systemic and mucosal infections than parental immunisation. However, it is not clear yet whether these protein-based vaccines would induce the same protective responses in humans. However, it is evident from the presented data that the antigens must be formulated into some delivery system, since several works have shown that plain antigens are not able to induce an adequate immune response. Simple nebulisation or administration of antigens without adjuvants resulted in low or no immune response at all (Table 1), and the new approaches based on nanomaterials and bacterial-like particles seem to be tendency now (Table 3), which might be related to the easier quality control, when compared to live recombinant bacteria (Table 2), for example, and less concerns about safety in comparison to toxoid adjuvants such as CTB or LT mutants (Table 1).

In addition to their adjuvant properties, other advantages are related to nanoparticle formulations. They can enhance antigen stability, direct the response to specific targets of the immune system, and allow preparation of dry-powder formulations that do not need cold-chain for storage, which should improve the access

to vaccination. Moreover, mucosal administration offers the advantage of eliminating syringes and needles, removing the hazard of safe disposal and lowering the risk of blood-borne infections.

Some important questions could be addressed experimentally in the next years, for example, if there are differences between nasal and pulmonary routes, how to guarantee the administration of the intended dose and if mucosal vaccination should be an alternative or a complementary strategy to the existing parenteral pneumococcal vaccines. The uncertainty regarding the translation from animal models to humans for mucosal vaccines is an essential force driving toward clinical trials. Although clinical trials are extremely necessary, there are several difficulties to perform them, mainly in places with universal PCV vaccination. It will be critical to define end-points for these trials to show non-inferiority in relation to current vaccines, taking into account other parameters than IPD, such as colonisation, reduction of upper and lower respiratory tract infections, the absence of serotype replacement, and the impact on microbiota. It is noteworthy that the definition of the end-points should face the lack of consensus for pneumonia diagnosis. The experimental human pneumococcal carriage (EHPC) and/or other human models are interesting alternatives to answer some of these questions.

Pathways for licensing mucosal vaccines are not as clear as for parenteral vaccines. The case of an inactivated pneumococcal whole cell vaccine is a paradigmatic one. This vaccine was originally developed to be given via the intranasal route [118], but intramuscular administration was applied in the clinical trial [39]. The hindrance may be related to the fact that antibody mediated immune response, which is the one normally induced by parenteral administration, is very well understood. It is thought that neutralising antibodies bind to the antigen and block the infection. For pneumococcal vaccines, antibodies that can induce opsonophagocytosis are considered correlates of protection, while cellular immune responses and mucosal immunity have not yet unequivocal correlates of protection.

Finally, the upcoming mucosal vaccine preparations could address issues important for developing countries such as storage, stability and ease of administration, aiming at developing cheaper vaccines that could reach the poorest people in the most remote locations.

Article highlights

- The existing pneumococcal vaccines are less effective at preventing pneumococcal pneumonia compared to IPD and serotype replacement has been mitigating the benefits of vaccination.
- Protein antigens could solve the serotype replacement problem and PspA is one of the most important antigens capable to induce protective immune response.
- Generation of local immune response could offer protection against pneumococcal colonisation and lung infection.
- In order to be administered into the lungs or intranasally, proteins have to be formulated in an adequate delivery system.
- The most promising delivery systems for mucosal administration are based on nanoparticles, bacterial-like particles or nanogels, as these formulations possess greater immunogenicity compared to the antigen alone and are considered safer than approaches based on living cells and adjuvanted with toxoids.
- The particles can protect the antigen from degradation, increase antigen uptake, control release of antigen and trigger innate immune responses.
- Particles can also stabilise the antigen, allowing the manufacture of dry powder formulations, which requires no refrigeration during storage.

Acknowledgments

The authors thank Sao Paulo Research Foundation and Medical Research Council for the joint grant FAPESP-MRC (FAPESP 2016/50143-8 and MR P022758 1) and BactiVac (grant BVNCP-02) for financial support. Liverpool John Moores University have filed a GB patent application to cover aspects of their work in this area (UK Patent Application Number: 1818517.3 - Nanoparticle Delivery System).

REFERENCES

* 1 Ramos-Sevillano E, Ercoli G, Brown JS. Mechanisms of naturally acquired immunity to *Streptococcus pneumoniae*. Frontiers in Immunology. 2019;10:358.

* The comprehension of the natural mechanisms of immunity against *S. pneumoniae* may help the development of new vaccines

2 Henriques-Normark B, Tuomanen EI. The pneumococcus: epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med. 2013;3(7):a010215.

3 GBD 2017 Causes of Death Collaborators. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 2018;392:1736-88.

4 Simell B, Auranen K, Käyhty H, et al. The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines. 2012;11(7):841–855.

5 Van Buynder P, Booy R. Pneumococcal vaccination in older persons: where are we today? Pneumonia. 2018;10:1.

6 Geno KA, Gilbert GL, Song JY, et al. Pneumococcal capsules and their types: past, present, and future. Clin Microbiol Rev. 2015;28(3):871–899.

7 Clutterbuck EA, Lazarus R, Yu LM, et al. Pneumococcal conjugate and plain polysaccharide vaccines have divergent effects on antigen-specific B cells. J Infect Dis. 2012;205(9):1408–16.

8 Papadatou I, Tzovara I, Licciardi PV. The role of serotype-specific immunological memory in pneumococcal vaccination: current knowledge and future prospects. Vaccines (Basel). 2019;7(1):E13.

9 WHO. Pneumococcal conjugate vaccines in infants and children under 5 years of age: WHO position paper – February 2019. Weekly epidemiological record. 2019;94(8):85–104.

10 Luna CM, Pulido L, Burgos D. Why is the rate of pneumococcal pneumonia declining? Curr Opin Pulm Med. 2018;24(3):205-211.

11 Rodrigo C, Bewick T, Sheppard C, et al. Impact of infant 13-valent pneumococcal conjugate vaccine on serotypes in adult pneumonia. Eur Respir J. 2015;45(6):1632-1641.

12 Fortunato F, Martinelli D, Cappelli MG, Cozza V, Prato R. Impact of pneumococcal conjugate universal routine vaccination on pneumococcal disease in Italian children. J Immunol Res. 2015;2015:206757.

*13 Waight PA, Andrews NJ, Ladhani SN, et al. Effect of the 13-valent pneumococcal conjugate vaccine on invasive pneumococcal disease in England and Wales 4 years after its introduction: an observational cohort study. Lancet Infect Dis. 2015;15:535–43.

* Very well documented analysis of the serotype replacement phenomenon.

14 Corcoran M, Vickers I, Mereckiene J, et al. The epidemiology of invasive pneumococcal disease in older adults in the post-PCV era. Has there been a herd effect? Epidemiol Infect. 2017;145(11):2390–9.

15 Savulescu C, Krizova P, Lepoutre A, et al. Effect of high-valency pneumococcal conjugate vaccines on invasive pneumococcal disease in children in SpIDnet countries: an observational multicentre study. Lancet Respir Med. 2017;5(8):648-656.

16 Hanquet G, Krizova P, Valentiner-Branth P on behalf of The SpIDnet/I-MOVE+ Pneumo Group, et al. Effect of childhood pneumococcal conjugate vaccination on invasive disease in older adults of 10 European countries: implications for adult vaccination. Thorax. 2019;74:473-482.

17 Mitsi E, Roche AM, Reiné J, et al. Agglutination by anti-capsular polysaccharide antibody is associated with protection against experimental human pneumococcal carriage. Mucosal Immunol. 2017;10:385-394.

18 Davis SM, Deloria-Knoll M, Kassa HT, et al. Impact of pneumococcal conjugate vaccines on nasopharyngeal carriage and invasive disease among unvaccinated people: review of evidence on indirect effects. Vaccine 2013;32:133-145.

19 O'Brien KL, Millar EV, Zell ER, et al. Effect of pneumococcal conjugate vaccine on nasopharyngeal colonization among immunized and unimmunized children in a community-randomized trial. J Infect Dis. 2007;196:1211-1220.

20 Dagan R, Givon-Lavi N, Fraser D, et al. Serum serotype-specific pneumococcal anticapsular immunoglobulin G Concentrations after immunization with a 9-valent conjugate pneumococcal vaccine correlate with nasopharyngeal acquisition of pneumococcus. J Infect Dis. 2005;192:367-376.

21 German EL, Solórzano C, Sunny S, et al. Protective effect of PCV vaccine against experimental pneumococcal challenge in adults is primarily mediated by controlling colonisation density. Vaccine 2019; doi:10.1016/J.VACCINE.2019.05.080

22 Dunne EM, Satzke C, Ratu FT, et al. Effect of ten-valent pneumococcal conjugate vaccine introduction on pneumococcal carriage in Fiji: results from four annual cross-sectional carriage surveys. Lancet Glob Heal. 2018;6:e1375-e1385.

23 Hanage WP, Finkelstein JA, Huang SS, et al. Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. Epidemics 2010;2:80-84.

24 Veenhoven R, Bogaert D, Uiterwaal C, et al. Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. Lancet 2003;361:2189-2195.

25 Miller E, Andrews NJ, Waight PA, et al. Herd immunity and serotype replacement 4 years after seven-valent pneumococcal conjugate vaccination in England and Wales: an observational cohort study. Lancet Infect Dis. 2011;11:760-768.

26 Ladhani SN, Collins S, Djennad A, et al. Rapid increase in non-vaccine serotypes causing invasive pneumococcal disease in England and Wales, 2000–17: a prospective national observational cohort study. Lancet Infect Dis. 2018;18:441-451.

27 Feikin DR, Kagucia EW, Loo JD, et al. Serotype-specific changes in invasive pneumococcal disease after pneumococcal conjugate vaccine introduction: a pooled analysis of multiple surveillance sites. PLoS Med. 2013;10:e1001517

28 Weinberger DM, Malley R, Lipsitch M. Serotype replacement in disease after pneumococcal vaccination. Lancet 2011;378:1962-1973.

29 Richter L, Schmid D, Kanitz EE, et al. Invasive pneumococcal diseases in children and adults before and after introduction of the 10-valent pneumococcal conjugate vaccine into the Austrian national immunization program. PLoS One 2019;14:e0210081.

30 Ciruela P, Izquierdo C, Broner S, et al. The changing epidemiology of invasive pneumococcal disease after PCV13 vaccination in a country with intermediate vaccination coverage. Vaccine 2018;36:7744-7752.

31 Hammitt LL, Etyang AO, Morpeth SC, et al. Effect of ten-valent pneumococcal conjugate vaccine on invasive pneumococcal disease and nasopharyngeal carriage in Kenya: a longitudinal surveillance study. Lancet 2019;393:2146-2154.

32 Sime WT, Aseffa A, Woldeamanuel Y, et al. Serotype and molecular diversity of nasopharyngeal *Streptococcus pneumoniae* isolates from children before and after vaccination with the ten-valent pneumococcal conjugate vaccine (PCV10) in Ethiopia. BMC Infect Dis. 2019;19:409.

33 Rupp R, Hurley D, Grayson S, et al. A dose ranging study of 2 different formulations of 15-valent pneumococcal conjugate vaccine (PCV15) in healthy infants. Hum Vaccin Immunother. 2019;15(3):549-559.

34 Stacey HL, Rosen J, Peterson JT, et al. Safety and immunogenicity of 15-valent pneumococcal conjugate vaccine (PCV-15) compared to PCV-13 in healthy older adults. Hum Vaccin Immunother. 2019;15(3):530-539.

35 Peterson JT, Stacey HL, MacNair JE, et al. Safety and immunogenicity of 15-valent pneumococcal conjugate vaccine compared to 13-valent pneumococcal conjugate vaccine in adults ≥65 years of age previously vaccinated with 23-valent pneumococcal polysaccharide vaccine. Hum Vaccin Immunother. 2019;15(3):540-548.

36 Hulten KG. The changing epidemiology of pneumococcal diseases. The Lancet Infectious Diseases. 2018;18(9):929-930.

37 Pichichero ME. Pneumococcal whole-cell and protein-based vaccines: changing the paradigm. Expert Rev Vaccines. 2017;16(12):1181-1190.

38 Prymula R, Szenborn L, Silfverdal SA, et al. Safety, reactogenicity and immunogenicity of two investigational pneumococcal protein-based vaccines: Results from a randomized phase II study in infants. Vaccine. 2017;35(35 Pt B):4603-4611.

**39 Campo JJ, Le TQ, Pablo JV, et al. Panproteome-wide analysis of antibody responses to whole cell pneumococcal vaccination. Elife. 2018;7:e37015.

** It provides information on how pneumococcal antibody repertoire develops in humans based on serum samples from phase I clinical trial.

40 Chan WY, Entwisle C, Ercoli G, et al. A novel, multiple-antigen pneumococcal vaccine protects against lethal *Streptococcus pneumoniae* challenge. Infect Immun. 2019;87(3):e00846-18.

41 Lagousi T, Basdeki P, Routsias J, Spoulou V. Novel protein-based pneumococcal vaccines: assessing the use of distinct protein fragments instead of full-length proteins as vaccine antigens. Vaccines (Basel). 2019;7(1):E9.

42 Perciani CT, Barazzone GC, Goulart C, et al. Conjugation of polysaccharide 6B from *Streptococcus pneumoniae* with pneumococcal surface protein A: PspA conformation and its effect on the immune response. Clin Vaccine Immunol. 2013;20(6):858-66.

43 da Silva MA, Converso TR, Gonçalves VM, Leite LCC, Tanizaki MM, Barazzone GC. Conjugation of PspA4Pro with capsular *Streptococcus pneumoniae* polysaccharide serotype 14 does not reduce the induction of cross-reactive antibodies. Clin Vaccine Immunol. 2017;24(8):e00118-17.

44 Odutola A, Ota MOC, Antonio M, et al. Immunogenicity of pneumococcal conjugate vaccine formulations containing pneumococcal proteins, and immunogenicity and reactogenicity of co-administered routine vaccines - A phase

II, randomised, observer-blind study in Gambian infants. Vaccine. 2019;37(19):2586-2599.

45 Spijkerman J, van Gils EJM, Veenhoven RH, et al. Carriage of *Streptococcus pneumoniae* 3 years after start of vaccination program, the Netherlands. Emerg Infect Dis.2011;17:584-91.

46 Bosch AATM, van Houten MA, Bruin JP, et al. Nasopharyngeal carriage of Streptococcus pneumoniae and other bacteria in the 7th year after implementation of the pneumococcal conjugate vaccine in the Netherlands. Vaccine. 2016;34:531-539.

47 Camilli R, Vescio MF, Giufrè M, et al. Carriage of *Haemophilus influenzae* is associated with pneumococcal vaccination in Italian children. Vaccine. 2015;33:4559-4564.

48 Spijkerman J, Prevaes SMPJ, van Gils EJM, et al. Long-term effects of pneumococcal conjugate vaccine on nasopharyngeal carriage of *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis*. PLoS One. 2012;7:e39730.

49 Biesbroek G, Wang X, Keijser BJF, et al. Seven-valent pneumococcal conjugate vaccine and nasopharyngeal microbiota in healthy children. Emerg Infect Dis. 2014;20:201-210.

50 Wiertsema SP, Kirkham L-AS, Corscadden KJ, et al. Predominance of nontypeable *Haemophilus influenzae* in children with otitis media following introduction of a 3 + 0 pneumococcal conjugate vaccine schedule. Vaccine. 2011;29:5163-5170.

51 Casey JR, Adlowitz DG, Pichichero ME. New patterns in the otopathogens causing acute otitis media six to eight years after introduction of pneumococcal conjugate vaccine. Pediatr Infect Dis J. 2009;29:1.

52 Eskola J, Kilpi T, Palmu A, et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med. 2001;344:403-409.

53 Nzenze SA, Shiri T, Nunes MC, et al. Temporal association of infant immunisation with pneumococcal conjugate vaccine on the ecology of Streptococcus pneumoniae, *Haemophilus influenzae* and *Staphylococcus aureus* nasopharyngeal colonisation in a rural South African community. Vaccine. 2014;32:5520-5530.

54 Lee GM, Huang SS, Rifas-Shiman SL, et al. Epidemiology and risk factors for *Staphylococcus aureus* colonization in children in the post-PCV7 era. BMC Infect Dis. 2009;9:110.

55 Navne JE, Koch A, Slotved H-C, et al. Effect of the 13-valent pneumococcal conjugate vaccine on nasopharyngeal carriage by respiratory pathogens among Greenlandic children. Int J Circumpolar Health. 2017;76:1309504.

56 WHO 2016. [cited 2019 Apr 20] https://www.who.int/news-room/fact-sheets/detail/pneumonia

*57 Feikin DR, Hammitt LL, Murdoch DR, O'Brien KL, Scott JAG. The enduring challenge of determining pneumonia etiology in children: considerations for future research priorities. Clin Infect Dis. 2017;64(suppl_3):S188-S196.

* Critical review about pneumonia diagnosis, important to understand the choice of end-points for clinical trials.

58 McCullers JA. Insights into the interaction between influenza virus and pneumococcus. Clin Microbiol Rev. 2006;19:571–82.

59 Greenberg D, Givon-Lavia N, Ben-Shimol S, Ziv JB, Dagan R. Impact of PCV7/PCV13 introduction on community-acquired alveolar pneumonia in children <5 years. Vaccine 2015;33:4623-4629.

60 Alicino C, Paganino C, Orsi A, et al. The impact of 10-valent and 13-valent pneumococcal conjugate vaccines on hospitalization for pneumonia in children: A systematic review and meta-analysis. Vaccine 2017;35:5776-5785.

61 Mackenzie GA, Hill PC, Sahito SM, et al. Impact of the introduction of pneumococcal conjugate vaccination on pneumonia in The Gambia: population-based surveillance and case-control studies. Lancet Infect Dis 2017; 17:965-73.

62 Ouldali N, Levy C, Minodier P, et al. Long-term association of 13-valent pneumococcal conjugate vaccine implementation with rates of community-acquired pneumonia in children. JAMA Pediatr. 2019;173(4):362-370.

63 Huss A, Scott P, Stuck AE, et al. Efficacy of pneumococcal vaccination in adults: a meta-analysis. CMAJ 2009; 180(1): 48-58.

64 Moberley S, Holden J, Tatham DP, et al. Vaccines for preventing pneumococcal infection in adults. Cochrane Database Syst Rev 2013;(1):CD000422.

65 Diao WQ, Shen N, Yu PX, et al. Efficacy of 23-valent pneumococcal polysaccharide vaccine in preventing community-acquired pneumonia among immunocompetent adults: A systematic review and meta-analysis of randomized trials. Vaccine 2016;34:1496-1503.

66 Schiffner-Rohe J, Witt A, Hemmerling J, et al. Efficacy of PPV23 in preventing pneumococcal pneumonia in adults at increased risk - a systematic review and meta-analysis. PLoS One 2016;11(1):e0146338.

67 Tin Htar M, Stuurman AL, Ferreira G, et al. Effectiveness of pneumococcal vaccines in preventing pneumonia in adults, a systematic review and meta-analyses of observational studies. PLoS One. 2017;12(5): e0177985.68 Bonten MJ, Huijts SM, Bolkenbaas M, et al. Polysaccharide conjugate vaccine against pneumococcal pneumonia in adults. N Engl J Med. 2015;372(12):1114-1125.

69 Suaya JA, Jiang Q, Scott DA, et al. Post hoc analysis of the efficacy of the 13-valent pneumococcal conjugate vaccine against vaccine-type community-acquired pneumonia in at-risk older adults. Vaccine. 2018;36(11):1477-1483.

70 Gessner BD, Jiang Q, Van Werkhoven CH, et al. A public health evaluation of 13-valent pneumococcal conjugate vaccine impact on adult disease outcomes from a randomized clinical trial in the Netherlands. Vaccine. 2018;May 31: S0264-410X(18)30763-1.

71 Pilishvili T, Bennett NM. Pneumococcal disease prevention among adults: strategies for the use of pneumococcal vaccines. Vaccine. 2015;33(Suppl.4):D60-D65.

72 McLaughlin JM, Jiang Q, Isturiz RE, et al. Effectiveness of 13-valent pneumococcal conjugate vaccine against hospitalization for community-acquired pneumonia in older US adults: a test-negative design. Clin Infect Dis 2018;67(10):1498-1506.

73 Prato R, Fortunato F, Cappelli MG, Chironna M, Martinelli D. Effectiveness of the 13-valent pneumococcal conjugate vaccine against adult pneumonia in Italy: a case–control study in a 2-year prospective cohort. BMJ Open 2018;8:e019034.

74 Vila-Corcoles A, Ochoa-Gondar O, de Diego C, et al. Evaluating clinical effectiveness of 13-valent pneumococcal conjugate vaccination against pneumonia among middle-aged and older adults in Catalonia: results from the EPIVAC cohort study. BMC Infect Dis 2018;18:196.

75 España PP, Uranga A, Ruiz LA, et al. Evolution of serotypes in bacteremic pneumococcal adult pneumonia in the period 2001–2014, after introduction of the pneumococcal conjugate vaccine in Bizkaia (Spain). Vaccine 2019;37:3840-3848.

76 Jose RJ, Brown JS. Adult pneumococcal vaccination: advances, impact, and unmet needs. Curr Opin Pulm Med. 2017;23:225–30.

77 Alpar HO, Somavarapu S, Atuah KN, Bramwell VW. Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and DNA delivery. Advanced Drug Delivery Reviews. 2005;57(3):411-30.

78 Zaman M, Chandrudu S, Toth I. Strategies for intranasal delivery of vaccines. Drug delivery and translational research. 2013;3(1):100-9.

79 Garg NK, Mangal S, Khambete H, Tyagi RK. Mucosal delivery of vaccines: role of mucoadhesive/biodegradable polymers. Recent Patents on Drug Delivery & Formulation. 2010;4(2):114-28.

80 Carroll EC, Jin L, Mori A, Muñoz-Wolf N, Oleszycka E, Moran HBT, et al. The vaccine adjuvant chitosan promotes cellular immunity via DNA sensor cGAS-STING-dependent induction of type I interferons. Immunity. 2016;44(3):597-608.

81 Paranjpe M, Müller-Goymann CC. Nanoparticle-mediated pulmonary drug delivery: a review. International Journal of Molecular Sciences. 2014;15(4):5852-73.

**82 Pati R, Shevtsov M, Sonawane A. Nanoparticle vaccines against infectious diseases. Frontiers in immunology. 2018;9:2224.

** Overview of nanoparticle application in vaccines.

83 Wang S, Liu H, Zhang X, Qian F. Intranasal and oral vaccination with protein-based antigens: advantages, challenges and formulation strategies. Protein Cell. 2015;6(7):480-503.

84 Wright AKA, Bangert M, Gritzfeld JF, et al. Experimental human pneumococcal carriage augments IL-17A-dependent T-cell defence of the lung. PLoS Pathog. 2013;9:e1003274.

85 Ferreira DM, Neill DR, Bangert M, et al. Controlled human infection and rechallenge with *Streptococcus pneumoniae* reveals the protective efficacy of carriage in healthy adults. Am J Respir Crit Care Med. 2013;187:855–864.

86 Jambo KC, Sepako E, Heyderman RS, Gordon SB. Potential role for mucosally active vaccines against pneumococcal pneumonia. Trends Microbiol. 2010;18:81–89.

87 Perez E, Bonilla FA, Orange JS, Ballow M. Specific antibody deficiency: controversies in diagnosis and management. Front Immunol. 2017;8:586.

88 Jambo KC, Sepako E, Fullerton DG, et al. Bronchoalveolar CD4+ T cell responses to respiratory antigens are impaired in HIV-infected adults. Thorax. 2011;66: 375–382.

89 Nakada T, Russell JA, Boyd JH, Walley KR. IL17A genetic variation is associated with altered susceptibility to Gram-positive infection and mortality of severe sepsis. Crit Care. 2011;15:R254.

*90 Gritzfeld JF, Wright AD, Collins AM, et al. Experimental human pneumococcal carriage. J Vis Exp. 2013;(72):50115. doi:10.3791/50115

* It includes a video showing the experimental human pneumococcal challenge

91 Wright AKA, Ferreira DM, Gritzfeld JF, Wright AD, Armitage K, Jambo KC, et al. Human nasal challenge with *Streptococcus pneumoniae* is immunising in the absence of carriage. Wessels MR, editor. PLoS Pathog. Public Library of Science; 2012;8: e1002622. doi:10.1371/journal.ppat.1002622.

92 Mitsi E, Carniel C, Jochems SP, et al. Cross-talk of alveolar macrophages and T-cells boosts the lung immunity post nasopharyngeal pneumococcal colonisation. Presented at: 11th International Symposium on Pneumococci and Pneumococcal Diseases. Melbourne, Australia, 15-19 April 2018. Available: https://web.kenes.com/klead/ISPPD18AbstractCD/ISPPD2018/data/HtmlApp/main.html#18

93 Wu H, Nahm MH, Guo Y, Russell MW, Briles DE. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage,

pulmonary infection, and sepsis with *Streptococcus pneumoniae*. J Infect Dis. 1997;175:839–846.

94 Yamamoto M, Briles DE, Yamamoto S, Ohmura M, Kiyono H, McGhee JR. A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. J Immunol. 1998;161:4115–21.

95 Miyaji EN, Oliveira MLS, Carvalho E, Ho PL. Serotype-independent pneumococcal vaccines. Cell Mol Life Sci. 2013;70:3303–3326.

**96 Rodrigues TC, Oliveira MLS, Soares-Schanoski A, et al. Mucosal immunization with PspA (Pneumococcal surface protein A)-adsorbed nanoparticles targeting the lungs for protection against pneumococcal infection. PLoS One. 2018;13: e0191692.

** The only publication targeting the lungs in mouse model of pneumococcal infection.

97 Ferreira DM, Darrieux M, Silva DA, et al. Characterization of protective mucosal and systemic immune responses elicited by pneumococcal surface protein PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. Clin Vaccine Immunol. 2009;16: 636–645.

98 Yu J, Li B, Chen X, Lu J, et al. Comparison of immunogenicity and protection of two pneumococcal protein vaccines based on PsaA and PspA. Infect Immun. 2018;86: e00916-17.

99 Nayak AR, Tinge SA, Tart RC, McDaniel LS, Briles DE, Curtiss R, 3rd. A live recombinant avirulent oral *Salmonella* vaccine expressing pneumococcal surface protein A induces protective responses against *Streptococcus pneumoniae*. Infect Immun. 1998;66:3744-3751.

100 Seo SU, Kim JJ, Yang H, et al. Effective protection against secondary pneumococcal pneumonia by oral vaccination with attenuated *Salmonella* delivering PspA antigen in mice. Vaccine. 2012;30:6816-6823.

*101 Frey SE, Lottenbach KR, Hill H, et al. A Phase I, dose-escalation trial in adults of three recombinant attenuated *Salmonella* Typhi vaccine vectors producing *Streptococcus pneumoniae* surface protein antigen PspA. Vaccine. 2013;31:4874-4880.

* Clinical trial with live attenuated bacteria producing PspA, example of the difficulties to translate animal results to humans.

102 Muralinath M, Kuehn MJ, Roland KL, Curtiss R, 3rd. Immunization with *Salmonella enterica* serovar Typhimurium-derived outer membrane vesicles delivering the pneumococcal protein PspA confers protection against challenge with *Streptococcus pneumoniae*. Infect Immun. 2011;79: 887-894.

103 Kuipers K, Daleke-Schermerhorn MH, Jong WS, et al. *Salmonella* outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization. Vaccine. 2015;33:2022-2029.

104 Oliveira ML, Areas AP, Campos IB, et al. Induction of systemic and mucosal immune response and decrease in *Streptococcus pneumoniae* colonization by nasal inoculation of mice with recombinant lactic acid bacteria expressing pneumococcal surface antigen A. Microbes and infection / Institut Pasteur. 2006;8: 1016-1024.

105 Hanniffy SB, Carter AT, Hitchin E, Wells JM. Mucosal delivery of a pneumococcal vaccine using *Lactococcus lactis* affords protection against respiratory infection. J Infect Dis. 2007;195:185-193.

106 Campos IB, Darrieux M, Ferreira DM, et al. Nasal immunization of mice with *Lactobacillus casei* expressing the Pneumococcal Surface Protein A: induction of antibodies, complement deposition and partial protection against *Streptococcus pneumoniae* challenge. Microbes and infection / Institut Pasteur. 2008;10:481-488.

107 Van Braeckel-Budimir N, Haijema BJ, Leenhouts K. Bacterium-like particles for efficient immune stimulation of existing vaccines and new subunit vaccines in mucosal applications. Frontiers in immunology. 2013;4:282.

*108 Lu J, Guo J, Wang D, Yu J, Gu T, Jiang C, et al. Broad protective immune responses elicited by bacterium-like particle-based intranasal pneumococcal particle vaccine displaying PspA2 and PspA4 fragments. Human Vaccines & Immunotherapeutics. 2019;15(2):371-80.

* Recent work showing a successful use of BLP for nasal immunisation of PspA.

109 Li B, Chen X, Yu J, et al. Protection elicited by nasal immunization with pneumococcal surface protein A (PspA) adjuvanted with bacterium-like particles against *Streptococcus pneumoniae* infection in mice. Microb Pathog. 2018;123:115–119.

110 Kye Y-C, Park S-M, Shim B-S, et al. Intranasal immunization with pneumococcal surface protein A in the presence of nanoparticle forming polysorbitol transporter adjuvant induces protective immunity against the *Streptococcus pneumoniae* infection. Acta Biomater. 2019;doi:10.1016/J.ACTBIO.2019.03.049.

*111 Kunda NK, Alfagih IM, Miyaji EN, Figueiredo DB, Goncalves VM, Ferreira DM, et al. Pulmonary dry powder vaccine of pneumococcal antigen loaded nanoparticles. International Journal of Pharmaceutics. 2015;495(2):903-12.

* Formulation and characterisation of a dry powder vaccine.

112 Xu J, Dai W, Wang Z, Chen B, Li Z, Fan X. Intranasal vaccination with chitosan-DNA nanoparticles expressing pneumococcal surface antigen A protects mice against nasopharyngeal colonization by *Streptococcus pneumoniae*. Clinical and vaccine immunology. CVI. 2011;18(1):75-81.

113 Ai W, Yue Y, Xiong S, Xu W. Enhanced protection against pulmonary mycobacterial challenge by chitosan-formulated polyepitope gene vaccine is associated with increased pulmonary secretory IgA and gamma-interferon(+) T cell responses. Microbiol Immunol. 2013;57(3):224-35.

114 Xu JH, Dai WJ, Chen B, Fan XY. Mucosal immunization with PsaA protein, using chitosan as a delivery system, increases protection against acute otitis media and invasive infection by *Streptococcus pneumoniae*. Scandinavian journal of immunology. 2015;81:177-185.

115 Kong IG, Sato A, Yuki Y, et al. Nanogel-based PspA intranasal vaccine prevents invasive disease and nasal colonization by *Streptococcus pneumoniae*. Infect Immun. 2013;81:1625–34.

116 Fukuyama Y, Yuki Y, Katakai Y, et al. Nanogel-based pneumococcal surface protein A nasal vaccine induces microRNA-associated Th17 cell responses with neutralizing antibodies against *Streptococcus pneumoniae* in macaques. Mucosal Immunol. 2015;8: 1144.

117 Trolle S, Chachaty E, Kassis-Chikhani N, et al. Intranasal immunization with protein-linked phosphorylcholine protects mice against a lethal intranasal challenge with *Streptococcus pneumoniae*. Vaccine. 2000;18:2991-2998.

118 Malley RM, Lipsitch A, Stack R, et al. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. Infect Immun. 2001;69:4870-4873.

119 Malley R, Srivastava A, Lipsitch M, et al. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. Infect Immun. 2006;74:2187-2195.

120 Jakobsen H, Saeland E, Gizurarson S, Schulz D, Jonsdottir I. Intranasal with pneumococcal polysaccharide conjugate vaccines protects mice against invasive pneumococcal infections. Infect Immun, 1999;67:4128-4133.

121 Jakobsen H, Schulz D, Pizza M, Rappuoli R, Jonsdottir I. Intranasal immunization with pneumococcal polysaccharide conjugate vaccines with nontoxic mutants of *Escherichia coli* heat-labile enterotoxins as adjuvants protects mice against invasive pneumococcal infections. Infect Immun. 1999;67:5892-5897.

122 Lynch JM, Briles DE, Metzger DW. Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. Infect Immun. 2003;71:4780-4788.

123 Menzel M, Muellinger B, Weber N, Haeussinger K, Ziegler-Heitbrock L. Inhalative vaccination with pneumococcal polysaccharide in healthy volunteers. Vaccine. 2005;23:5113-5119.

124 Meyer P, Menzel M, Muellinger B, Weber N, Haeussinger K, Ziegler-Heitbrock L. Inhalative vaccination with pneumococcal polysaccharide in patients with chronic obstructive pulmonary disease. Vaccine. 2006;24:5832-5838.

* 125 Gordon SB, Malamba R, Mthunthama N, et al. Inhaled delivery of 23-valent pneumococcal polysaccharide vaccine does not result in enhanced pulmonary mucosal immunoglobulin responses. Vaccine. 2008;26:5400-5406.

* It illustrates the need of an adequate formulation to deliver antigens to the lungs.

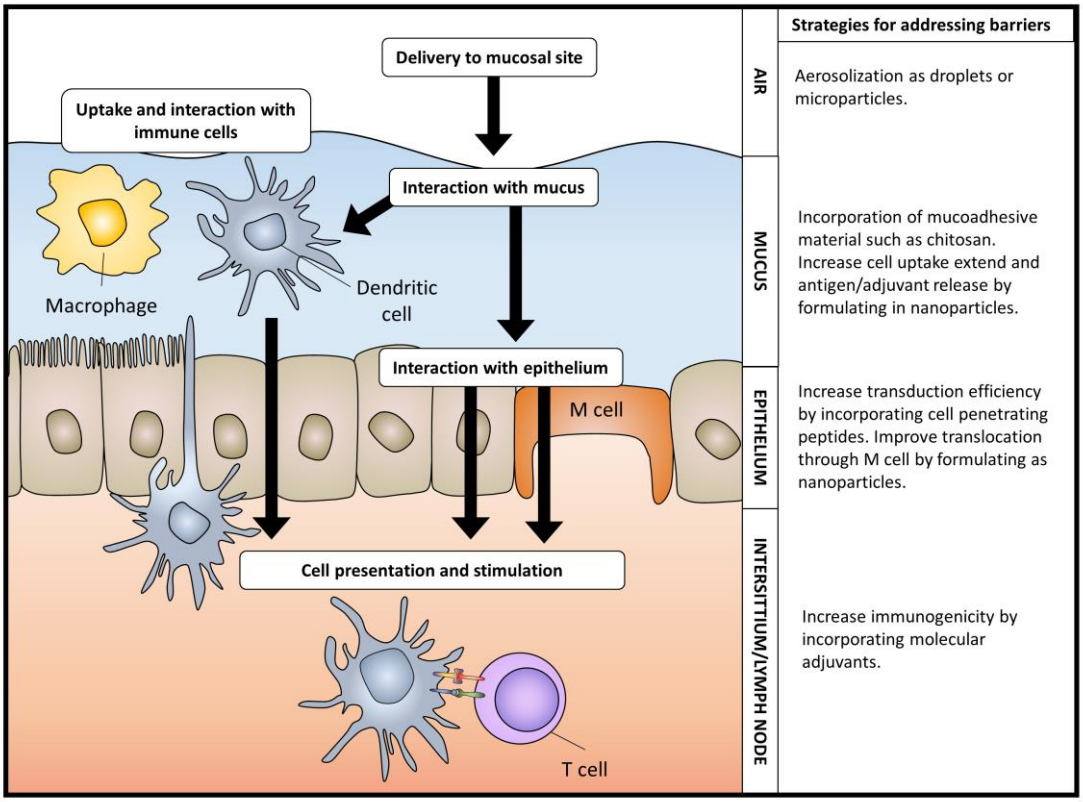

Figure 1: Overview of the barriers associated with vaccine delivery at the mucosal surface after administration through the nasal or pulmonary route. Common strategies for improving the vaccine efficacy through overcoming identified barriers have been listed.

Table 1 – Early attempts for mucosal immunisation against pneumococcal diseases: use of pneumococcal antigens with adjuvants

Vaccine candidate	Administration route	Model	Protection	Reference
PspA + CTB[a]	nasal	murine	protection against fatal intratracheal and intravenous challenges Reduction of pneumococcal load in nasal wash	[93]
PspA + nontoxic CTA[b]	nasal	murine	higher protection against fatal intravenous challenge than with native CT[c]	[94]
PS1-TT[d] and PS3-TT[e] + RhinoVax[f]	nasal	murine	protection against lung infection and bacteraemia caused by serotype 1 80% survival after fatal intranasal challenge with serotype 3	[120]
PS1-TT[d] and PS3-DT[g] + LT-K63[h] and LT-R72[i]	nasal	murine	protection against lung infection and bacteraemia caused by serotype 1 ≥90% survival after fatal intranasal challenge with serotype 3	[121]
PS3-CRM_{197}[j] and PS14-CRM_{197}[k] + IL-12	nasal	murine	lower survival after intraperitoneal challenge with serotype 3 strain than intramuscular immunisation reduction of nasopharyngeal colonisation after challenge with a serotype 14 strain	[122]
phosphorylcholine conjugated to porcine thyroglobulin	nasal	murine	protection against lethal intraperitoneal challenge	[117]
inactivated whole cell	nasal	murine rat	protection against nasopharyngeal colonisation reduced morbidity and mortality after intrathoracic challenge with serotype 3 strain	[118]
cell wall polysaccharide + CTB[a] or CT[c]	nasal	murine	increase resistance to nasopharyngeal colonisation protection against fatal aspiration pneumonia	[119]
inhaled PPV23[m]	pulmonary	healthy human volunteers and patients with COPD[n]	lower levels of serum antibodies when compared to intramuscular immunization	[123,124]
inhaled PPV23[m]	pulmonary	healthy human volunteers	no increase of IgG and IgA in serum and BALF[o]	[125]

[a] CTB – cholera toxin B subunit; [b] CTA - cholera toxin A subunit; [c] CT – cholera toxin; [d] PS1-TT – pneumococcal polysaccharide from serotype 1 (PS1) conjugated to tetanus toxoid (TT); [e] PS3-TT – pneumococcal polysaccharide from serotype 3 (PS3) conjugated to TT; [f] RhinoVax – adjuvant formulation based on caprylic-capric glycerides dissolved in polysorbate 20 and water; [g] PS3-DT – PS3 conjugated to diphtheria toxoid (DT); [h] LT-K63 and [i] LT-R72 – mutants of heat-labile enterotoxin (LT) of *Escherichia coli*; [j] PS3-CRM_{197} – PS3 conjugated to nontoxic mutant of CRM_{197} diphtheria toxin; [k] PS14-CRM_{197} – pneumococcal polysaccharide from serotype 14 (PS14) conjugated to CRM_{197}; [m] inhaled 23-valent pneumococcal polysaccharide vaccine (PPV23) contains no adjuvant; [n]COPD – chronic obstructive pulmonary disease; [o] BALF – bronchoalveolar lavage fluid.

Table 2 – Live recombinant vaccines for mucosal immunisation against pneumococcal diseases

Vaccine candidate	**Administration route**	**Model**	**Protection**	**Reference**
attenuated *Salmonella* Typhimurium expressing PspA	oral	murine	survival after fatal intraperitoneal challenge	[99]
attenuated *Salmonella* Typhimurium expressing PspA	oral	murine	survival after fatal intratracheal challenge	[100]
attenuated *Salmonella* Typhi expressing PspA	oral	healthy human volunteers	no increase in anti-PspA titers in phase I dose escalation clinical trial	[101]
lactobacilli and *Lactococcus lactis* expressing PsaA	nasal	murine	reduction of pneumococcal load in nasal wash after lactobacilli immunisation, but no reduction with *L. lactis*	[104]
L. lactis expressing PspA	nasal	murine	protection against lethal intraperitoneal and respiratory challenges	[105]
L. casei expressing PspA	nasal	murine	partial protection against lethal intraperitoneal challenges	[106]

Table 3 – New approaches for mucosal immunisation against pneumococcal diseases

Vaccine candidate	Administration route	Model	Protection	Reference
Salmonella OMV-PspA	nasal	murine	complete protection against low dose and partial against high dose intraperitoneal challenge	[102]
Salmonella OMV-PspA(α-1 α-2)[a]	nasal	murine	dose-dependent reduction of pneumococcal load in nasal tissue	[103]
Salmonella OMV-PspA(LFBD-PRR)[b]	nasal	murine	no reduction of pneumococcal load in nasal tissue	[103]
BLP-PspA-PA fusion[c]	nasal	murine	80-90% survival after fatal challenge	[98]
BLP-PspA3-PA fusion[c]	nasal	murine	100% survival after fatal challenge	[109]
BLP-PspA2-PA + BLP-PspA4-PA fusions[c]	nasal	murine	100% survival after intranasal fatal challenge with 2 pneumococcal strains	[108]
Chitosan-*psa*A gene	nasal	murine	reduction of nasopharyngeal colonisation	[112]
Chitosan-PsaA	nasal	murine	clearance of pneumococcus from middle ear protection against intraperitoneal challenge	[114]
cCHP-PspA[d]	nasal	murine	reduction of colonization and invasion in upper and lower respiratory tracts100% survival after fatal challenge	[115]
cCHP-PspA[d]	nasal	macaques	passive immunisation protects mice from intravenous fatal challenge	[116]
PGA-co-PDL-PspA[e]	pulmonary	murine	lower pneumococcal load in BALF 67% survival after fatal challenge	[96]
PST-PspA[f]	nasal	murine	100% survival after fatal challenge	[110]

[a] α-helical coiled coil domains (α-1 and α-2) of PspA; [b] lactoferrin-binding domain (LFBD) and the Pro-rich region (PRR) of PspA; [c] fusion of PspA with the protein anchor (PA), C-terminus of lactococcal protein ACMA, which serves to attach the protein to BLP; [d] cationic cholesteryl pullulan (cCHP) nanogel; [e] PGA-co-PDL - poly(glycerol adipate-co-ω-pentadecalactone); [f] polysorbitol transporter (PST), which is made up of sorbitol diacrylate and low-molecular-weight polyethylenimine.