Chitosan nanocarrier systems for delivery of pneumococcal vaccine via nebulization

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Summary

Background: *Streptococcus pneumoniae* enters the human body via the lungs and is the main cause of pneumonia. The current vaccine is administered via the parenteral route and has limited protection in the lungs and towards the different strains of *S. pneumoniae*. Pulmonary vaccination is an attractive alternative targeting dendritic cells (DCs) in the lungs initiating an immune response. To optimise targeting and uptake by DCs, chitosan (CHT) and chitosan hydrochloride (CHCl) nanoparticles (NPs) were loaded with pneumococcal surface protein A (PspA) and delivered via nebulization for pulmonary delivery.

Methods: CHT/PspA and CHCl/PspA NPs were prepared by ionic gelation method. The formulations were then collected by centrifugation (35,000rpm, 10min, 25°C) to remove unbound PspA and re-suspended in 1mL of 0.9% NaCl for nebulization. NPs were characterised in terms of particle size, surface charge, polydispersity index (PDI), drug loading after centrifugation, post nebulization dose, particle morphology, *in vitro* release and cell viability (DCs) after 24h.

Results: Results indicated the size of CHT/PspA NPs (236.36±5.57nm, +10.78±0.47mV, PDI 0.366±0.045), CHCl/PspA NPs (372.633±7.02nm, +31.8±1.16mV, PDI 0.216±0.025) were suitable for targeting DCs. PspA loading after centrifugation and nebulization (CHT/PspA NPs: 9.92µg/mg,9.69 µg/mg), and (CHCl/PspA NPs: 1.62µg/mg, 1.29 µg/mg) indicated little loss. The release studies revealed continuous release of protein after 24h (CHT/PspA NPs: 26%, CHCl/PspA NPS: 99%). The NPs appear to be well tolerated by DCs.

Conclusion: The results indicated chitosan NPs could be a promising candidate for pulmonary delivery of pneumococcal vaccine.

Introduction

The increase in mortality and morbidity, mainly correlated with pulmonary diseases, such as pneumonia, has gained significant attention to produce a non-invasive approach to enhance immunogenity [1]. The main portal of entry of *Streptococcus pneumoniae* into the body is the respiratory tract. Among the non-invasive routes of delivery, the pulmonary route can overcome some challenges such as invasiveness, low stability and integrity of the antigen. The current vaccine is administered via the parenteral route and has limited protection towards the different strains of *S. pneumoniae* and in the lungs. A new formulation is required to achieve higher efficacy and level of protection against mucosal diseases [2].

In order to generate a stronger immune response, particulate antigens have been preferred to soluble antigen, and research has focused on using nanoparticles (NPs) as a delivery carrier and potential adjuvant. In fact, NPs of appropriate size (150–500 nm) and charge (−40 mV - +35 mV) are able to enter the lymphatics and travel to DCs within the lymph nodes, while larger particles (> 500 nm) are not so efficient and selective [3]. Surface charge of NPs are known to play an important role in determining the cellular uptake, and cationic NPs compared to anionic or neutral, have better interactions with the negatively charged cell membrane [1]. Nebulization of aqueous NPs suspensions seems to be an appropriate delivery mechanism to achieve deposition of NPs in the lungs to target DCs [4].

Chitosan has attracted particular interest as a biodegradable material for mucosal delivery systems, especially for its important capacity to enhance drug permeability and absorption at mucosal site [5]. Despite all its positive biological properties, it has a major drawback; it is soluble in acidic conditions, which may affect the stability and integrity of some antigens. As an alternative, chitosan can be functionalised for the synthesis of chitosan hydrochloride (CHCl), a water soluble derivative that retains its mucohadesive and uptake properties, and can be considered as a promising candidate for vaccine delivery.

Aim

The aim of this study was to prepare and compare different chitosan NPs (chitosan in acetic acid (CHT) and chitosan hydrochloride (CHCl)) complexed pneumococcal surface protein A (PspA) in terms of nebulization for targeting lung DCs.

Methods:

Nanoparticle preparation

Chitosan NPs were prepared by ionic gelation method. Briefly, 10 mg of CHT (Sigma, UK) were dissolved in 5 ml of 1% acetic acid solution, while 10 mg of CHCl (Heppe Medical Chitosan GmbH, Halle, Saale) were dissolved in 5 ml of water. The pH was adjusted to 6 using NaOH 0.5M. Subsequently, various amount of TPP aqueous solution with the ratio CHT:TPP 3:1, with PspA (0.1 mg/ml) incorporated in TPP were added drop-by-drop to the above solutions under magnetic stirring (250 RPM) at room temperature for 1 hour. The colloidal suspension was centrifuged at 35,000 rpm for 10 min at 25 °C and washed twice with distilled water to remove unbound PspA.

Nanoparticle characterization

Particle size, Zeta-Potential and Polydispersity index (PDI): the NPs were characterised using a laser particle size analyser (ZetasizerNano ZS, Malvern Instruments Ltd., UK). 1 ml of NPs suspensions was loaded into a cuvette and the measurements were recorded at 25 °C (mean ± SD, n=3).

Drug loading (DL): PspA loaded in the NPs was determined by measuring the amount of protein remaining in the supernatant after centrifugation, using a QuanicPro bicinchoninic acid (BCA) protein assay kit (micro BCA assay, Sigma-Aldrich) by UV spectroscopy at 562 nm (mean ± SD, n=3). Empty CHT and CHCl NPs were used as a control.

Post nebulization dose: Loaded NPs after centrifugation were resuspended in 1 ml of 0.9% NaCl and nebulized for 5 minutes using Aerogen Pro Lab Nebulizer (Aerogen, Galway, Ireland), a vibrating mesh nebulizer with multiple apertures to generate fine-particles and low velocity aerosol. The post nebulization dose was calculated to determine the compatibility of the nebulizer with the chitosan NPs/PspA formulation.

Particle morphology: 50 µl of loaded NPs were mounted on gold coated aluminium stubs (EmiTech K 550X Gold Sputter Coater, 25mA for 3 min) and visualised by scanning electron microscopy (SEM).

In vitro Release studies: Loaded NPs after centrifugation were dispersed in 2 ml of PBS, pH 7.4. The samples were incubated at 37 °C and left rotating at 20 RPM on a HulaMixer™ Sample Mixer (Life Technologies, Invitrogen, UK). The samples were then centrifuged after 30 min, 1, 2, 4 and 24 hours at 17,000 x g for 10 min and 1 ml of the supernatant was removed and replaced with fresh medium. The supernatant was analysed with micro BCA assay.

Cell Viability study: The toxicity of the NPs were investigated in DCs (ATCC, JAWS II) using MTT assay [2]. The treatment was carried out for 4 hours at 37 °C, followed by the addition of MTT for 2 hours. 100 µl PspA NPs dispersions in complete medium were added at concentration (0.001–1 mg/ml) and 10% dimethyl sulfoxide (DMSO) as a positive control. The absorbance was measured at 570 nm using a plate reader (Molecular Devices, SpectraMAX 190) and the percentage of viable cells was calculated as the absorbance between NPs complexed with PspA and untreated control DCs.

Results and Discussion

The ionic gelation method produced particles in the nanometer size range with positive zeta potential values, which are associated with the free amine groups on the chitosan following complexation with TPP (Table 1). Loaded NPs were larger in size than blank NPs, indicating encapsulation of PspA. Moreover, loaded NPs revealed a lower charge than blank NPs. This was due to the presence of the protein, which has a negative charge when the pH (6) is above its pI (4.9).

Table1 - The Particle size, PDI and zeta potential for CHT and CHCl non-loaded (blank) and loaded nanoparticles before nebulization (mean ± SD, n=3).

	Particle Size (nm)	***PDI***	***Z-Potential (mV)***
CHT NPs	206.70 ± 3.75	0.370 ± 0.02	+11.9 ± 0.48
CHT/PspA NPs	236.36 ± 5.57	0.366 ± 0.05	+10.78 ± 0.47
CHCl NPs	347.00 ± 3.24	0.189 ± 0.014	+33.20 ± 1.13
CHCl/PspA NPs	372.63 ± 7.02	0.216 ± 0.025	+31.80 ± 1.16

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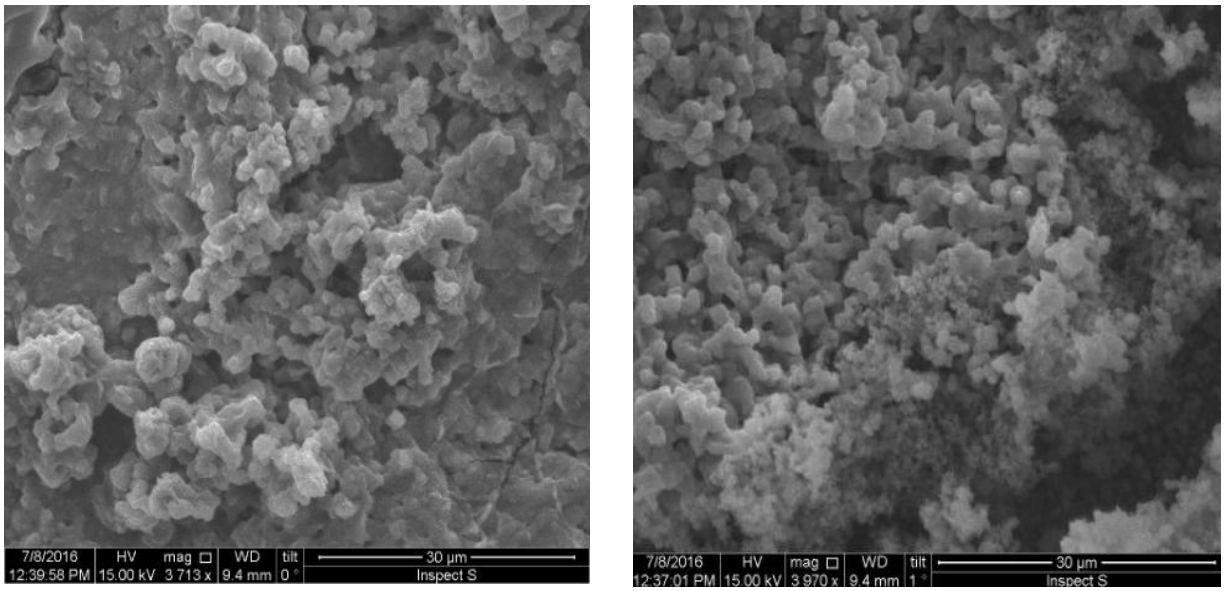
The micro BCA assay after centrifugation and nebulization confirmed the high drug loading (DL) and aerosolised dose in each formulation of CHT and CHCl, as shown in Table 2

Table 2 - Drug loading (DL) of PspA NPs after centrifugation and aerosolised dose after nebulization

	Concentration of protein (µg/ml)	**DL after centrifugation (µg/10 mg NPs)**	**Post nebulization dose (µg/10 mg NPs)**
CHT/*PspA NPs*	100	99.20	96.90
CHCl/PspA NPs	20	16.24	12.98



A B

Figure 1 - SEM pictures of CHCl non-loaded NPs (A) loaded (B) NPs after nebulization. Scale bar 30 µm.

Owing to their small size and low inertia, NPs are commonly exhaled after inhalation resulting in low doses in the lungs. To overcome this problem, the NPs have been nebulized in order to achieve the appropriate characteristics of size suitable for the pulmonary delivery. In fact, it is known that particles in the size range of 1 to 5 µm are required to reach the respirable airways. SEM analysis (Figure 1) of non-loaded (A) and loaded (B) nanoparticles after nebulization revealed spherical NPs with rough surface morphology. The particle sizes observed by SEM were pproximately 1 µm, indicating the NPs were aggregated post nebulization.

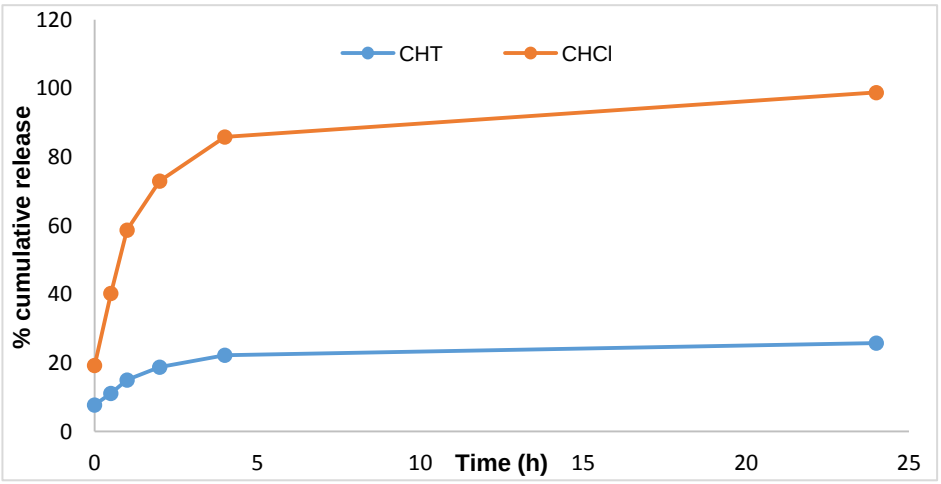


Figure 2 - *In vitro* release of PspA from CHT (A) and CHCl (B) NPs in phosphate buffer saline (pH 7.4) over 24 hours at 37 °C.

CHT and CHCl NPs showed significant difference in release of PspA. The cumulative percentage of PspA released over time from CHT and CHCl NPs (Figure 2) indicated an initial burst release of 8% (CHT) and 20% (CHCl), followed by continuous release of 26% (CHT) and 99% (CHCl) after 24 hours. The much higher burst release of PspA from CHCl NPs rather than the CHT NPs, may be due to the higher charge of the polymer and the water-soluble properties which could result in faster dissolution and release of PspA.

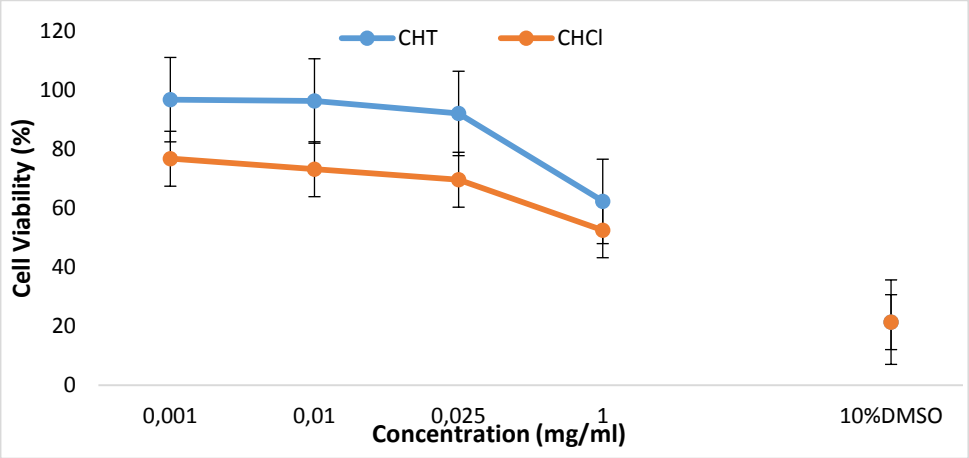


Figure 3 - Dendritic cells (DCs) viability measured by MTT assay after 24 hours exposure to NPs.

The cell viability of DCs incubated with CHT/PspA and CHCl/PspA NPs for 4 hours was evaluated by the MTT assay. The NPs (Figure 3) revealed reduced cell viability with an increasing NPs concentration. The NPs showed a 97% cell viability for CHT and 77% for CHCl at 0.001 mg/ml concentration that reduced to 63% (CHT) and 52% (CHCl) at 1 mg/ml concentration. The lower cellular viability of CHCl may be due to the higher positive zeta potential that has been attributed to the interactions with the plasma membrane.

Conclusion:

The results obtained indicated high PspA loading in the CHT and CHCl formulations, with almost no loss post nebulization. The high delivery efficiency of the vibrating mesh nebulizer has the advantage of reducing the vaccine dose and to improve the cost-effectiveness compared to the conventional ultrasonic nebulizers. In addition, our results indicated CHT and CHCl have a low *in vitro* cell toxicity towards DCs which is required for pulmonary drug delivery carriers. Further studies would focus on the *in vivo* immune studies.

References:

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