Nano-in-microparticle powders for mucosal vaccination – understanding the particle forming process

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Summary

Nanoparticulate antigen encapsulation to induce a local immune response upon nasal application is a promising alternative to the commonly used parenteral vaccination. In this study, the particle forming process of such nanoparticles, consisting of chitosan and a low viscosity sodium carboxymethylcellulose sodium salt – produced via ionic gelation, is illustrated. Ovalbumin as model antigen was incorporated into the particles for some experiments. Different qualities of chitosan (varying degree of deacetylation as well as molecular weight) were investigated regarding their influence on the resulting particle sizes, showing an increase of size for qualities with a higher molecular weight. For selected qualities a closer look into the particle forming process was taken to improve its understanding. For this, gel permeation chromatography and ^{1}H-NMR experiments were performed to assess whether changes in the chitosan regarding molecular weight or degree of deacetylation occur during particle formation, to get an idea about the "reactive share" of the chitosan used. To gain further information regarding the binding mechanisms between the components isothermal titration calorimetry experiments were performed finding differences between the different chitosan qualities. Moreover, a different counterion (sodium deoxycholate) was utilised to detect potential differences depending on the counterion (large carboxymethylcellulose sodium salt, approximately 90 kDa vs. small sodium deoxycholate, 0.4 kDa) regarding the particle size as well as the zeta potential. Nanoparticles can be incorporated in a microparticulate stabilising matrix to allow respiratory application and deposition.

Introduction

Mucosal vaccination via the respiratory tract holds promise and has been under research for some time [1]. Both, the nasal and the pulmonary mucosa, are easily accessible and comprise many immune cells, making it an especially attractive application route. Furthermore, the respiratory tract is the natural site of entrance for many pathogens and therefore an even more promising target for vaccination. Still, there are challenges associated with this as well, i.e. a sufficient dwell time on the mucosa as well as a sufficient uptake into and activation of antigen presenting cells.

Antigens encapsulated into particulate systems are able to induce a local immune response in addition to a systemic one [1]. Moreover, particulate systems have shown superior immune effects compared to dissolved antigen. The uptake furthermore depends on the particle size. Those ranging from 250 to 400 nm are most favourable [2]. Therefore, in this project a nano-in-microparticle-formulation (NiM) has been chosen as vehicle because the nanoparticle ensures the uptake into cells whereas the microparticle (produced via spray drying of the nanosuspension with mannitol as matrix) secures the deposition on the mucosa (10-50 µm particles for deposition on the nasal mucosa [3] and particles smaller than 5 µm for pulmonary deposition).

In this study the focus lies on the understanding of the nanoparticle forming process. Hence, nanoparticles based on chitosan and two different counterions (large carboxymethylcellulose sodium salt and small sodium deoxycholate) have been produced and analysed.

Materials and Methods

Preparation of nanoparticles

Different chitosan qualities (with varying degree of deacetylation (DDA) and molecular weight) obtained from Heppe Medical Chitosan, Germany, were used. A low viscosity carboxymethylcellulose sodium salt (CMC, approximately 90 kDa, Sigma-Aldrich, USA) and sodium deoxycholate (DOC, 0.4 kDa, Carl Roth GmbH + Co. KG, Germany) served as counterions. Ovalbumin (OVA, Sigma-Aldrich, USA) was used as a model antigen.

Nanoparticles were formed by ionic gelation of the positively charged chitosan with the negatively charged counterion (CMC or DOC). Chitosan was dissolved in acetic acid (2 % (w/v) for particle formation with CMC and 1 % (w/v) with DOC, respectively) to a concentration of 0.1 %. CMC or DOC was dissolved in ultrapure water (Direct-Q 3 UV, Merck Millipore, Germany) to the same concentration. Nanoparticles formed spontaneously upon mixing of these two components utilising a magnetic stirrer.

Size and zeta potential measurements

Size and zeta potential of the nanoparticles were determined with the Zetasizer Nano ZS (Malvern Instruments, UK). Size measurements (utilising dynamic light scattering) were performed with undiluted nanosuspensions whereas the zeta potential (utilising Laser Doppler anemometry) was measured with a 1:2 diluted suspension. Each measurement was performed in triplicate, results are given as mean.

Determination of molecular weight

Gel permeation chromatography (GPC) served to determine the molar mass using a PL-GPC 50 Plus (Polymer Laboratories, USA). The system contains an integrated degasser and a differential refractive index detector (Polymer Laboratories, Varian Inc., USA). A MALS detector (Mini DAWN Tristar, Wyatt) was utilised for the determination of molar mass, assuming a refractive index increment of 0.185 mL/g [4]. For the separation by size, a PL aquagel OH Guard 8 µm precolumn followed by a PL aquagel OH 40 8 µm column and one PL aquagel OH 30 8 µm column (Agilent Technologies, Santa Clara, USA) in series were used. Samples (3 mg/mL in acetic acid 1 %) were eluted with 0.25 M $NaNO_3$ und 0.01 M NaH_2PO_4 in water (pH 2.3) at a flow rate of 1.0 mL/min and the column temperature was kept at 35 °C.

Determination of DDA

^{1}H-NMR was utilised to assess the DDA of chitosan. The experiment was performed modifying the method of Lavertu et al. [5]. Samples were dissolved in 2 %CD_3COOD (Carl Roth GmbH + Co. KG, Germany) to a concentration of 5 mg/mL. The measurements were performed with a BRUKER Avance III 300 MHz spectrometer (Bruker Corporation, USA) at 80 °C. The DDA was determined using Equation 1:

$$DDA = \left(\frac{H_1 D}{H_1 D + \frac{H_{Ac}}{3}}\right) \times 100\ \% \qquad \text{Equation 1}$$

Determination of binding characteristics

Typically, isothermal titration calorimetry (ITC) is used to characterise the binding affinities between proteins and ligands. In our study we used the MicroCal VP-ITC (Malvern Instruments, UK) to gain knowledge about the interaction between chitosan and CMC. For this chitosan and CMC were dissolved in 1 % acetic acid, chitosan to a concentration of 0.05 % in the cell and CMC to 0.9 % in the syringe (=injectant). For some experiments OVA was added to the reaction, either in the syringe (0.44 %) or in the cell (0.05 %). 35 injections (8 µL over 16 s, spaced by 200 s) were made per experiment. Stirring speed was set at 307 rpm. Measurements were performed at 25 °C.

Results and Discussion

Particle sizes obtained when using different qualities of chitosan for the process are shown in Figure 1. Size can be tuned by using different molecular weight chitosan, whereas the DDA does not influence the particle size. Further, particles get slightly smaller upon the incorporation of antigen compared to unloaded particles (data not shown). As target particle size is between 250 and 400 nm to ensure efficient uptake by immune competent cells, the molecular weight should be kept below 150 kDa.

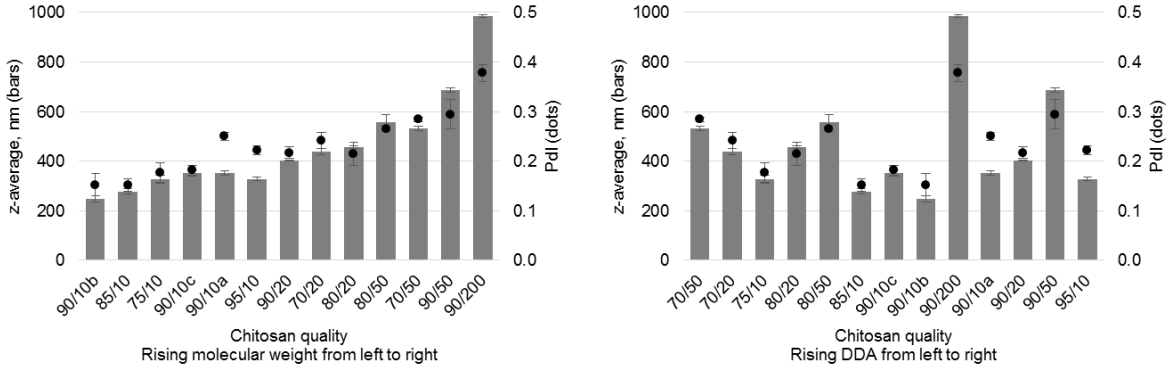

Figure 1 – Resulting nanoparticle sizes utilising different chitosan qualities (first set of numbers represents the DDA in percent, second set the viscosity in mPas (1 % solution in acetic acid 1 %, 20 °C); for the chitosan 90/10 three different batches (a-c) have been utilised). On the left the different chitosans have been ordered according to their molecular weight, on the right according to their DDA. (n=3, error bars=sd)

During nanoparticle processing it became obvious that the polymer does not completely react to nanoparticles. To better understand the particle forming process, DDA and molar mass were determined before and after particle formation. For these experiments it was necessary to obtain unreacted chitosan after particle formation; consequently, a nanosuspension was centrifuged to separate the nanoparticles from the supernatant. The excess chitosan in the supernatant was precipitated using 1 N NaOH prior to repeated centrifugation. The chitosan was dried for 48 h in a vacuum drier subsequently.

Figure 2 shows the results for the ^{1}H-NMR as well as the GPC experiments. DDA and molar mass were lower after particle formation compared to the starting substances. To conclude, the "more reactive chitosan" was found to have both a higher molecular weight and a higher DDA (meaning more free amino groups). Nevertheless, no separate fractions of molecular weight could be found for the examined chitosan qualities. The respective peak simply shifts slightly after particle formation. Hence, it was not possible to identify the "reactive part" of the chitosan to work with this exclusively. The same is true for the DDA, a "reactive part" could not be separated from the rest.

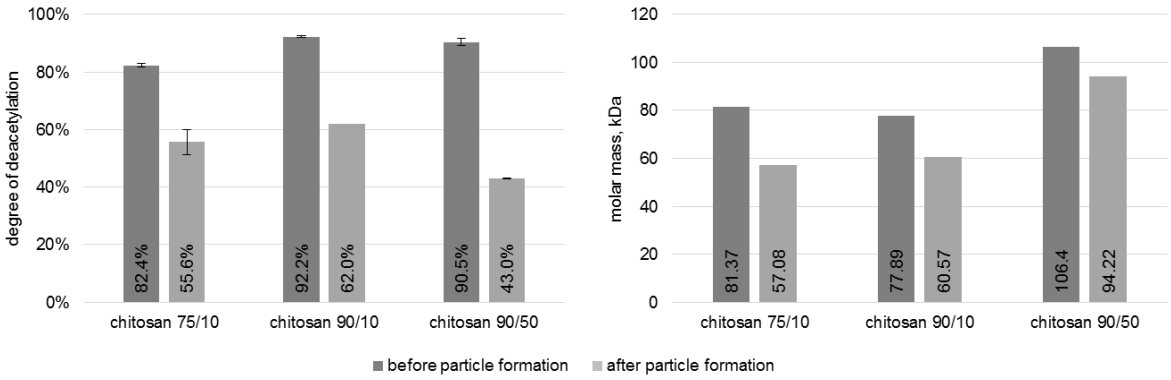

Figure 2 – Left: Results from ^{1}H-NMR experiments showing the DDA values for the different chitosan qualities before and after particle formation. Error bars show standard deviation. Right: Resulting molar mass values for the different chitosan qualities before and after particle formation.

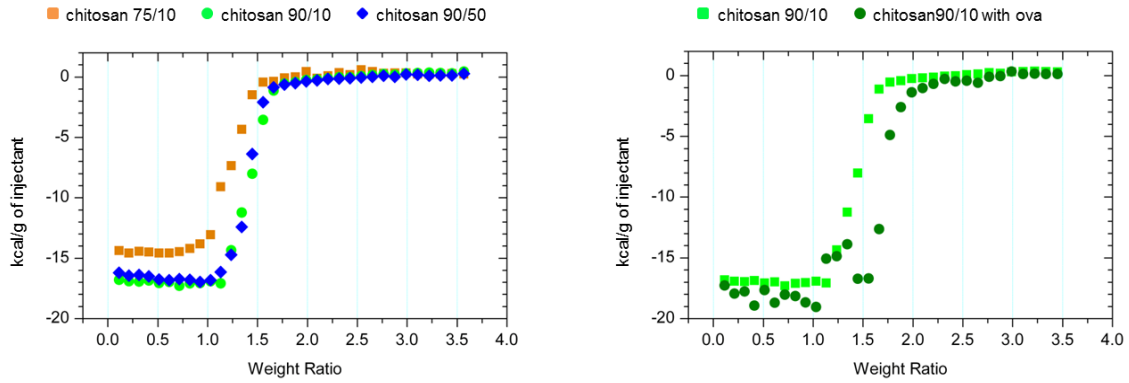

Figure 3 – ITC results. Left: Binding curves for different chitosan qualities reacting with CMC showing a difference in binding affinities between varying DDA and no difference for different molecular weights. Right: Differences between particle formation including or not including OVA in the cell displaying that more CMC is necessary for the reaction if additional cations (OVA) are present. The concentration of the OVA is equal to the concentration of chitosan but not taken into account on the axis.

Isothermal titration calorimetry experiments showed that no reaction took place between chitosan and OVA which was expected because both polymers have a positive charge in acidic medium. Further experiments (Figure 3) showed differences in the particle formation process: Results suggest that a higher DDA led to more chitosan reacting with the CMC as well as OVA in the chitosan phase resulted in an increased amount of reacting CMC. Molecular weight however does not influence the reaction kinetics. Problem with these experiments was the titration to the "endpoint" – in the case of chitosan nanoparticles this meant agglomeration as the nanosuspension is only stabilised electrostatically.

Consequently, if all the positive charges of the chitosan are neutralised, agglomeration occurs. Formulations with a zeta potential larger than +30 mV (or smaller than -30 mV) are considered stable [6]. Figure 4 shows the results of the titration of CMC to chitosan 90/10 – either containing or not containing OVA. It could be shown clearly that at a certain ratio of the two ions (chitosan and CMC) z-average increases dramatically. At the same time, zeta potential drops below 30 mV indicating loss of stabilisation and agglomeration of nanoparticles. In this experiment

no apparent difference in zeta potential was observed irrespective of the formulation including OVA or not. Accordingly, the difference observed using ITC is probably rather small and not relevant for the particle formation on a larger scale.

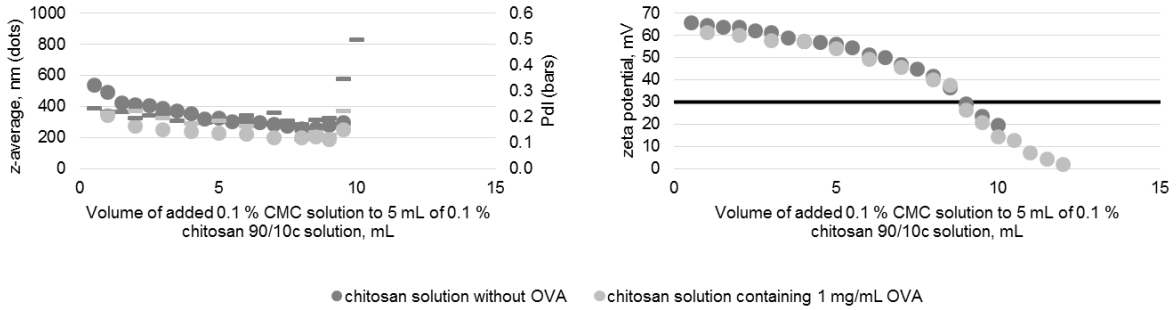

Figure 4 – Left: Resulting nanoparticle sizes from titration experiment with either placebo- or OVA-containing NP. Right: Resulting zeta potentials for the nanosuspensions.

These findings were compared to particles formed with chitosan and DOC showing (Figure 4) a difference in particle formation between the two counterions. The smaller DOC formed smaller particles with a higher zeta potential most likely due to a lower charge density. Further experiments need to be performed to verify this hypothesis.

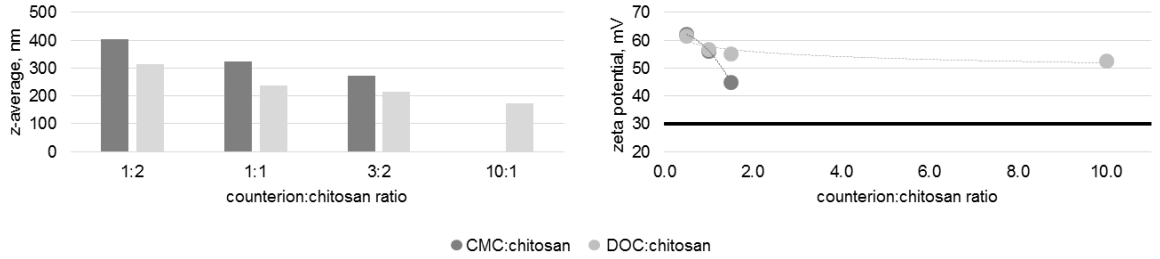

Figure 5 – Differences in particle size (left) and zeta potential (right) of nanosuspension formed with CMC and DOC, respectively.

Conclusion and Outlook

The particle forming process for chitosan:CMC nanoparticles could be elucidated with the chosen methods. The nanoparticle size could be tuned by the molecular weight of chitosan, an increasing molecular weight resulted in larger particles. The experiments examining molecular weight and DDA of the chitosan before and after particle formation showed that these parameters change during the process and therefore it is not simple to determine the amount of chitosan reacted as most quantification methods depend on the DDA. The ITC experiments further showed an influence of the DDA on the particle forming process as a higher DDA resulted in a more intense reaction (in terms of energy and binding ratio). For the counterion both the type as well as the amount influences particle size and zeta potential. Future work will be focussing on the protein encapsulation efficiency for different formulations as well as their efficiency regarding uptake into and activation of immunocompetent cells.

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