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Original Article

Encapsulation of Inactivated Newcastle Disease Virus Onto the Chitosan Nanoparticles for Use in Mucosal Immunity

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Abstract

Background and Aims: Newcastle disease virus (NDV) as the causative agent of a serious respiratory infection threatens the poultry industry worldwide. The risk of an outbreak of NDV could be restricted by mass vaccination. Here we brought up methodology to generate chitosan (CS)-based NDV nanoparticles as an antigen carrier for delivery into intranasal mucosa.

Materials and Methods: The NDV antigen was produced in the allantoic cavity of 9-11-day embryonated SPF chicken eggs. The virus was tittered and inactivated by ethylene imine. The CS nanoparticles were prepared by ionic gelation method. The physicochemical properties including size, distribution, charge, and morphology of the particles were evaluated. The CS-based NDV nanoparticles were prepared by encapsulation of the inactivated NDV antigen and validated based on the factors affecting optimal encapsulation. The toxicity and safety of the nanoparticles were assayed using inoculation of HLM cells as well as by administration of SPF chickens.

Results: The CS nanoparticles were produced with an average size of 196 nm and good morphology. After encapsulation of NDV antigen, the mean diameter of the nanoparticles was 328 nm with an encapsulation efficiency of ~83% and loading capacity of ~54%. The electron microscopy study indicated that the particles had a spherical shape. The in vitro cytotoxicity and the in vivo safety of the CS-based NDV nanoparticles results indicated the particles were not toxic either in LMH cells or in chickens.

Conclusion: By considering factors that represent optimal nanoparticles, the safe inactivated NDV nanoparticles were successfully developed. This study lays the foundation for the further development of mucosal vaccines and drugs encapsulated in chitosan nanoparticles. By considering factors that represent optimal nanoparticles, the safe inactivated NDV nanoparticles were successfully developed. These results provide a foundation for the further development of mucosal vaccines based on inactivated antigens for use in chicken.

Keywords: Newcastle disease; Chitosan; Nanoparticles; Encapsulation

Introduction

Avian infectious diseases have emerged as a serious threat to the poultry industry. Among the diseases, Newcastle disease (ND) causes an enormous impact on the health care and management sectors. The viral respiratory disease of birds caused by virulent strains of the ND virus (NDV) a member of Paramyxo-viridae family in the genus avian avulavirus-1. The negative-stranded RNA genome of NDV encodes six major proteins in order to 5'-L, HN, F, M, P, NP-3'. The virus enters the host through the mucosal tissues of the respiratory tract, disseminates towards other organs, and

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transmits horizontally through airborne droplets or exposure to virus contaminated fomites or through mechanical vectors [1]. The NDV strains are classified into the diverse types based on their virulence; velogenic neurotropic and velogenic viscerotropic strains, mesogenic, lentogenic and asymptomatic strains [2].

Establishing an adequate vaccination program using the lentogenic live attenuated vaccines and inactivated oil-emulsion vaccines is the principal strategy to control the disease in countries in which virulent NDV strains are endemic in order to reduce the substantial economic losses of the disease [3]. Despite several advantages of the vaccines, their protective efficacy is threatened by vaccine-related factors. Molecular epidemiology data show that the virulent strains of NDV are still circulating worldwide causing substantial morbidity and mortality in chickens and backyards [4-8]. The occurrence of outbreaks and the emergence of the new NDV genotypes, administration of multiple immunizations that increase the cost of vaccination, underline a need for the development of more effective vaccines against the disease. Consequently, attention has now been focused on two categories; the generation of genotype-matched vaccines and the improvement of vaccine antigen delivery [9-12].

The first prerequisite step of the NDV infection occurs at epithelial of the respiratory tract. The virus enters the cell through the direct fusion of the viral envelope with the plasma membrane or through caveola-mediated endocytosis. This is commonly associated with low-pH-dependent activation of the viral F protein [13, 14]. Thus, the good defense at the mucosa is critical for the prevention of virus replication and further systemic spread. The major challenge is the efficient delivery of vaccines to the host immune cells. One of the plausible approaches could be the application of nanoparticle vaccines due to their minimal side effects and require fewer doses over classical vaccines. The mucosal delivery of these vaccines can induce both mucosal and systemic immune responses. For two decades chitosan (CS) is considered as a carrier for whole particle, protein, and DNA-based vaccines in the mucosal delivery system [12, 15-17]. The mucoadhesive biopolymer comprises repeating units of b-(1-4)-2-amino-2-deoxy-D-glucopyranose is found to be non-toxic, biodegradable, and biocompatible. The increased aqueous solubility of the conjugated hydrophobic moiety in the delivery platform and high loading capacity of CS for various biological agents facilitate the development of CS-based nanoparticles [18]. The related researches indicate that the CS-based nanoparticles enhance antigen uptake by mucosal lymphoid tissues lead to the induction of strong immune responses against the vaccine antigen. The live NDV antigen encapsulated in CS nanoparticles induced a higher secretary IgA antibody response in the intestinal mucosa of chickens and conferred protection against highly virulent NDV challenge [19]. This result is not unexpected compared to live NDV vaccines administered via spray, drinking water or eye-drop methods. But due to the limited immunogenicity of inactivated vaccines that administrated intramuscularly, either high antigenic dose or a booster dose are needed. By considering the advantages of mucosal immunity in protection against respiratory viruses' infections, we used CS nanoparticles for the encapsulation of inactivated NDV antigen. We, here, focused on the preparation and optimization of CS-based NDV nanoparticles to ensure the better physico-chemical properties and stability of the vaccine candidate.

Methods

NDV antigen preparation and titration: The NDV LaSota strain was inoculated into the allantoic cavity of 9-11-day embryonated SPF eggs and incubated at 37°C and 5% humidity for 7 days. The allantoic fluid was harvested and examined for sterility and haemagglutination activity according to OIE Manual [2]. To test for possible bacterial contamination, 0.2 ml of the collected fluid was placed into ten individual vessels each containing at least 120 ml of Soybean Casein Digest Medium and incubated at 30°to 35°C

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for 14 days. To test for fungi, the same amount of the sample was placed into ten individual vessels each containing at least 40 ml of Soybean Casein Digest Medium and incubated at 25°C for 14 days. The harvested allantoic fluid containing NDV was titrated in 10-day-old SPF embryonated eggs. Serial 10 fold dilution of the virus was carried out using sterile phosphate buffer saline. 0.1 ml of each dilution from 10-1 to 10-9 was inoculated in allantoic cavity of eggs, using at least five eggs per dilution. The eggs were incubated up to 7 days and the virus titer was calculated according to Reed and Muench and expressed in terms of 50% embryo infectious dose (EID50).

NDV antigen inactivation: The harvested allantoic fluid was inactivated with binary ethylenimine (BEI) [20]. Briefly, 2.55 g of bromo ethylamine (BEA) was added to 100 ml of 0.175 N sodium chloride to prepare BEI. The mixture was placed at 37°C for one hour to obtain a cyclic BEI. The 0.1 M BEI solution was mixed with the prepared NDV antigen and incubated at 30°C for 21 hours to complete the inactivation process. The remaining BEI was hydrolyzed by the addition of sodium thiosulfate. The inactivated antigen was tested to ensure the inactivation procedure. Three subsequent passages were carried out by inoculating the inactivated antigen in 10-day-old SPF embryonated eggs. The allantoic fluid of each passage was examined for HA activity .

Chitosan nanoparticles preparation and characterization: In the ionic gelation method, the low molecular weight chitosan (CS, Sigma-Aldrich) was dissolved in an aqueous solution of acetic acid to obtain a 0.5 mg/mL final concentration. The same concentration of sodium tripolyphosphate (TPP, Merck) was prepared in ultrapure water. These solutions separately passed through a syringe filter (0.22 μm, Millipore). The CS nanoparticles were prepared by dropping ice-cold TPP solution to the CS solution at ratios1:1 to 5:1under a magnetic stirrer at room temperature.

Particles size, polydispersity index, and charge of the synthesized CS nanoparticles were measured using Dynamic light scattering (DLS) with Malvern Zetasizer Nano ZS (Worcestershire, UK). The particle morpho-logy was measured using a transmission electron microscope (TEM; Zeiss-EM10C-100 KV, Germany). Fourier-transform infrared spectroscopy (FTIR) spectrum was recorded in the range of 400-450 cm-1 with FTIR-S 8400 (Shimdzu, Japan).

CS-based NDV nanoparticles preparation and validation: The CS-based NDV nanoparticles were prepared using an ionic cross-linking method. Each of 0.5, 1.0, and 2.0 ml of the prepared NDV inactivated antigen was mixed by the optimized ratio of CS/TPP solution under magnetic stirring at room temperature. The physicochemical features of the samples were analyzed as explained above. Encapsulation efficiency (EE) and loading capacity (LC) of the nanoparticles were determined by centrifugation of the solution at 10,000 g/min for 30 min at 4°C.The amount of free NDV and free CS in the supernatant were estimated using a spectrophotometer (CECIL-series 1000-LABOQUIMIA) at 596 nm.

The EE and LC of the nanoparticles were calculated from: EE% = (X0 – X1)/X0×100 and LC% = X0/(XCS+X0)/×100; where X0 is the total amount of NDV added, X1 is the amount of free virus, and XCS is the amount of free CS.

CS-based NDV nanoparticles cytotoxicity and safety determination: The chicken hepatocellular carcinoma cell line (LMH) at a density of 1 × 105 cells/mL in DMEM medium was seeded in a 96-well microplate. After 24 h incubation, cells in each well were exposed to the 100 µl of serial dilutions of CS-based NDV nanoparticles. The plate was incubated at 37°C in a humidified incubator containing 5% CO2 for 48 h. Then, the medium carefully removed and 10 µL of MTT reagent (5 mg/mL in PBS) and 100 µL DMEM were added to each well, and LMH cells were incubated for a further 4 h. The absorbance of the solution in each well was measured at 570 nm using a microplate reader. The relative cell viability was calculated by:

$$[A]\text{control} - [A]\text{test}/[A]\text{control} \times 100.$$

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Table 1. Size, zeta potential and dynamic light scattering of nanoparticles in different ratios of CS/TPP (n=3)

CS/TPP concentration (mg/mL)	Particle size (nm) (Mean ± SD)	Zeta potential (mV) (Mean ± SD)	Polydispersity index (Mean ± SD)
1:1	159 ± 6.28*	12.4 ± 6.48*	0.261 ± 0.051
1:2	196 ± 11.38*	13.6 ± 2.55*	0.175 ± 0.022
1:3	243.2 ± 31.26*	14.7 ± 4.64*	0.394 ± 0.134
1:4	379.8 ± 7.58*	15.6 ± 6.23*	0.404 ± 0.028
1:5	414.8 ± 10.47*	15.6 ± 2.37*	0.438 ± 0.017

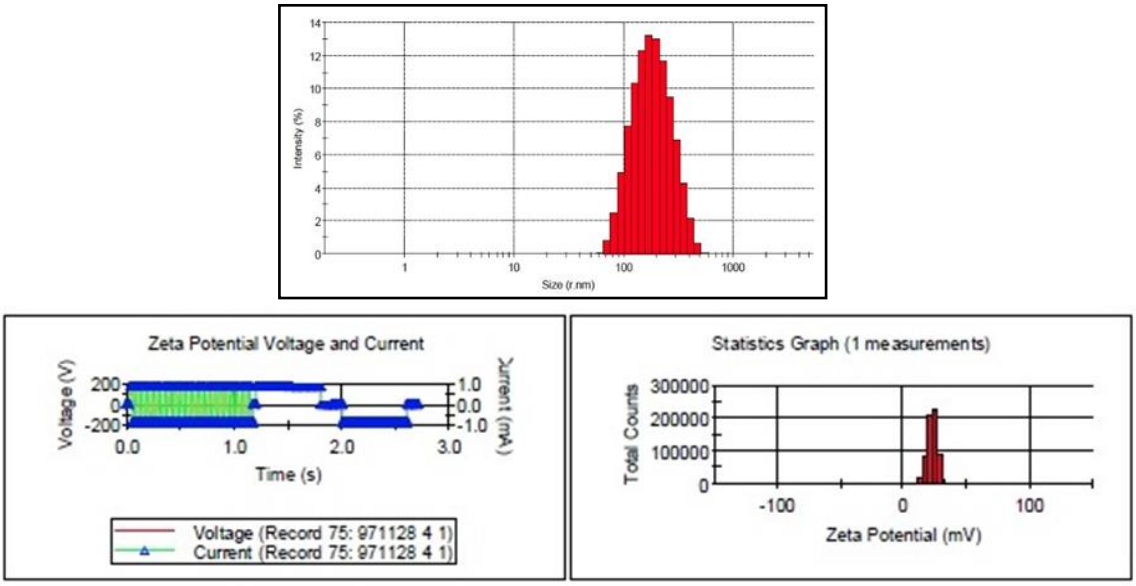

Fig. 1. Particle size distribution (up) and zeta potential (down) of the CS-based NDV nanoparticles under the optimized conditions

At in vivo trial, 20 SPF chickens at 4-week-old of age were randomly divided into two groups. Chickens in group A were received 0.25 ml of CS-based NDV nanoparticles by eye drop and the other group was kept as control. The chickens were examined for any abnormal signs for 21 days.

Results

The allantoic fluids of embryonated chicken eggs inoculated with NDV were collected. At a titer of $10^{9.67}EID_{50}$/ml, the harvested antigen was inactivated with BEI. The BEI-treated NDV antigen was checked for inactivation. After three subsequent passages, no HA titer was detected in the collected allantoic fluids indicating that the viral antigen was completely inactivated. Using ionic gelation methode, the CS nanoparticles were synthesized. Quantitative analysis of CS/TPP ratios is summarized in table 1. As tabulated in the table, the optimal nanoparticles were produced in a ratio of 1: 2. A similar profile was done for the preparation of CS-based NDV nano-particles and their physicochemical properties were analyzed. With the average particle sizes of 328 ± 9.6 nm with a polydispersity index of 0.251 ± 0.08, zeta potential 23.5 ± 4.78 mV (Fig. 1), EE 83.10 ± 1.28% and LC 54.20%± 0.18% the optimal combination was obtained.

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The CS nanoparticles and CS-based NDV nanoparticles displayed spherical morphology, smooth surfaces with clear edges, and good dispersion (Fig. 2).

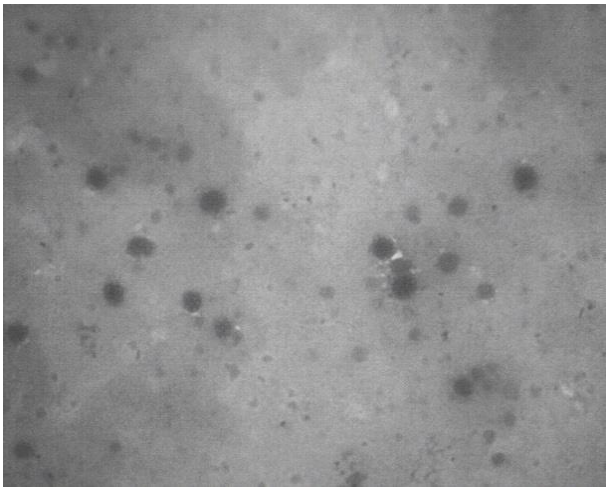

Fig. 2. Transmission electron microscopy of the CS-based NDV nanoparticles. The photomicrograph showed that the prepared nanoparticles had a regular round shape and good dispersion.

The nanoparticles sizes were in unison with the DLS size spectra. The FTIR spectrum for the prepared nanoparticles was recorded in the range of 4500-450 cm-1 (Fig. 3).

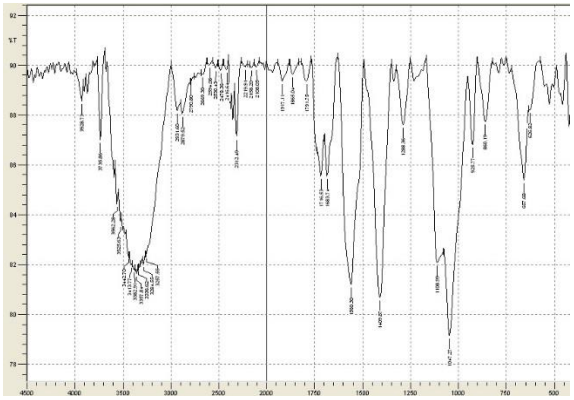

Fig. 3. The FTIR spectrum for the CS-based NDV nanoparticles showed the competitive displacement after loading of NDV antigen onto the CS nanoparticles.

This spectrum has free amine, hydroxyl, and ether groups. The peaks of the 3700-3100 region are related to the water in the nanoparticles, the 2500-2000 region are related to the C-H atomic bonds, and the 1615 peak is related to the tensile strength of the C-O groups and the R-CO-NH2 groups. Peaks of areas less than 1000 related to TPP and peaks 1542 indicate the interaction between TPP phosphate groups and chitosan amine groups. The little shift and increased relative intensity of peaks in CS-based NDV nanoparticles compared to the CS nanoparticles indicates the competitive displacement after loading of NDV.

The cytotoxicity analysis is essential to support the safety of nanoparticles. In this study, the in vitro cytotoxicity of CS-based NDV nanoparticles was evaluated in LMH cells using MTT assay. After 48h incubation of LMH cells with the nanoparticles, the survival rate of the cells was estimated at 90.18%±2.35%, and no cytopathic effects were observed compared to the control cell (Fig. 4).

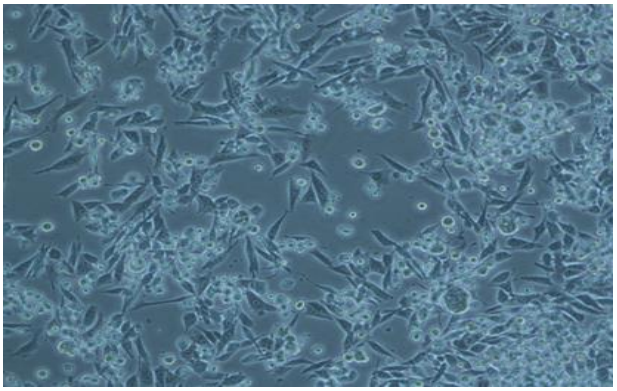

Fig.4. Cytotoxicity assay of the CS-based NDV nanoparticles in chicken LMH cells (X40). The high viability of the cells and the lack of cytopathic effects indicate the safety of the nanoparticles.

At in vivo analysis of the CS-based NDV nanoparticles, no clinical signs were observed in chickens until 3 weeks post-inoculation. The results showed that the prepared nanoparticles possess a high safety level for administration via the mucosal delivery system.

Discussion

Inactivation by BEI has first introduced about 40 years ago and now is used for the inactivation of many viruses in the development of inactivated vaccines procedure. The inactivator agent does not interfere with the virus structure at a low concentration. Most vaccine antigens inactivated properly by BEI can protect the host after challenge [21]. But in mucosal immunization, an inactivated virus is

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often insufficiently effective due to the inadequate intake of antigen by the nasal mucosa. Therefore, encapsulated vaccine antigens have been studied as a potent strategy to overcome the restriction. In this study, in order to develop an effective carrier as a delivery system for poultry vaccines, we prepared CS nanoparticles containing the inactivated NDV antigen using an ionic gelation method. The results showed that the inactivated NDV antigen was successfully encapsulated in CS nanoparticles and non-toxic for use in chicken.

The ionic gelation technique is suggested for vulnerable molecules such as the vaccine antigen. But many factors are affect the suitable formulating of nanoparticles for use as a vaccine include concentrations of each CS and TPP and their ratio in formation of the colloid [22]. In our study, the best CS and TPP combination with good physicochemical properties was achieved at CS/TPP ratio of 1:2. Then, in the same profile, CS nanoparticles containing inactivated NDV antigen were prepared. The CS-based NDV nanoparticles showed regular round shapes and good dispersion. The polydispersity is basically a representation of the distribution of size populations. The numerical value of the polydispersity index range from 0.0 to 1.0 indicates a perfectly uniform sample to a highly polydisperse with multiple particle size populations. The measured dispersity of CS-based NDV nanoparticles (0.2815] confirmed the uniformity of the size within this sample. The encapsulation of NDV in the nanoparticles resulted in increased zeta potential from 13.6 to 23.5 mV, which could be explained by the large size of the virus particle and interactions between the antigen and the CS nanoparticles. This size differences indicate the proper loading of antigen onto CS nanoparticles. The optimal combination between CS nanoparticles and NDV antigen was yielded at high EE 83% and LC 54%. The loading interaction was also assessed by employing FTIR. The results showing a high percentage of EE and the formation of stable nanoparticles. It has been suggested that the rate of incorporating the antigen into nanoparticles determines the effectiveness of its delivery into the target cells of the nasal cavity [23].

Various factors are affecting antigen transport across the nasal epithelium following the mucosal vaccination including mucociliary clearance, phagocytosis, biological and/or chemical degradation of the vaccine antigen [24]. Chicken nasal-associated lymphoid tissue (NALT) as an inductive site of the upper respiratory tract mainly consists of a second lymphoid follicle. The NALT plays an essential role in the local immune activation because of a large number of lymphocytes distributed under the mucosal epithelium of the inferior nasal [25]. Considering the nasal absorptive capacity will be critical in nasal vaccination and the development of mucosal vaccines for poultry [26]. Thus the use of nanoparticles for vaccine delivery could be advantageous due to the fact that small particles can be transported preferentially by the NALT. Cationic polymers like CS with positive charges and low-viscosity have the potential to interact with the mucosal barriers can enhance the absorption of antigen molecules and protect it from degradation. The interaction between amino and carboxyl groups of positively charged CS with the negatively charged glycoproteins in in mucus produces an adhesive effect. In this regard, retention of the bioadhesive particles on the mucosal surface could contribute to enhanced nasal absorption leads to prolongation of the antigen release time and induction of desirable immune responses. The large epithelial surface in birds and mucoadhesive property of CS elicit both mucosal and systemic immune responses against entrapped antigens after intranasal administration. The positive charge of CS and the negative charge of antigen increase the bioadhesive property, which has a direct effect on the mucosal immunization efficiency [27, 28]. So, it is expected that the positively charged CS-based NDV nanoparticles could bind with the mucosal glycoproteins and also dendritic cells as the main antigen-presenting cells. This binding results in antigen delivery into the cytoplasm where antigen could be processed via the cytosolic pathway to induce immune responses against the pathogen.

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The LMH cell toxicity showed that the prepared CS-based NDV nanoparticles had no cytotoxicity in vitro. To test the in vivo safety, SPF chickens were received the CS-based NDV nanoparticles intranasal. The result indicates the CS-based NDV nanoparticles are safe because no NDV clinical signs were observed during immunization period. Overall, the CS-based NDV nanoparticles have the potential as an effective carrier for the antigen delivery in mucosal administration. Our result are similar to the other studies that shown intranasal vaccination by each of encapsulated live NDV, influenza subunit/split, and F gene DNA plasmid of NDV could improve mucosal IgA response in the respiratory tract, elicited humoral and cell mediated immune responses, and provided better protection [29, 17, 19].

Conclusion

A linear relationship between the physico-chemical properties of nanoparticles and an antigen provides a potential platform for their application in mucosal immunity. Despite the anticipated results, there are no reports on the commercial production of avian nanoparticle vaccines.

This comprises some questions in the formulation of nanoparticles, the relative toxicity of some polymers and other impurities, high cost, difficulties in controlled release of nanoparticles, as well as their relative ability to deliver immunogen to antigen-presenting cells. These points are the main challenges that will resolvable with advancements in biotechnology.

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Conflict of interest

The authors declare that they have no conflict of interest.

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