Colloids and Surfaces B: Biointerfaces 116 (2014) 9–16

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Formulation of Newcastle disease virus coupled calcium phosphate nanoparticles: An effective strategy for oculonasal delivery to chicken

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ARTICLE INFO

Article history:
Received 17 August 2013
Received in revised form
28 November 2013
Accepted 2 December 2013
Available online 30 December 2013

Keywords:
Nanoparticle
Calcium phosphate
Immune responses
Virus delivery
Cyclodextrin

ABSTRACT

In this report, calcium phosphate (CaP) nanoparticles were synthesized by continuous flow method using β-cyclodextrin (β-CD) as a medium and functionalized with amino propyl triethoxy silane (APTES). The blood biocompatibility of the nanoparticles was assessed using the whole blood haemolysis, erythrocytes haemolysis and erythrocyte aggregation tests. Based on the results, we found that the synthesized β-CD–CaP nanoparticles did not cause any remarkable toxic effect. The 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide (MTT) assay of chicken peripheral blood mononucleated cells (PBMCs) incubated with these nanoparticles indicated that these particles did not exert any significant cytotoxicity. The aminosilane functional group modified β-CD–CaP was used as tool for coupling of Newcastle disease virus (NDV). The NDV conjugated nanoparticles were confirmed by using Fourier transformed infrared spectroscopy, X-ray diffraction patterns, Raman spectroscopy differential scanning calorimetry and energy-dispersive X-ray spectroscopy. Immunogenicity trials in chickens proved that β-CD–CaP-NDV used as a vaccine was better than the commercial vaccine when given oculonasally during the first 2 weeks post vaccination. The birds vaccinated with the above nano-NDV vaccine were completely protected against virulent NDV challenge. This study confirms that the oculonasal β-CD–CaP-NDV delivery of vaccines is a potential method for enhancing the immune responses of existing commercial vaccines.

1. Introduction

One of the limitations of new generation vaccines in comparison with their live attenuated/killed counterparts is their reduced immunogenicity. In this context, nanoparticles-based vaccine delivery systems offer greater potential as it increases the bio availability of the antigens in the circulatory system [1–3]. The concentration of nanoparticles in the blood vessels is controlled by its size; it also has important effects on their extravasations and clearance mechanisms. Nanoparticles larger than 200 nm are eliminated by phagocytosis and those smaller than 5 nm are rapidly removed though renal clearance [4–6]. In recent years, researchers have paid more attention on calcium phosphate (CaP) nanoparticles and their biological applications. The Food and Drug Administration (FDA) has already certified that CaP nanoparticles are safe and bioresorbable [7]. Some of the biological applications using CaP nanoparticles include gene transfection [8], drug delivery [9,10], vaccine delivery [11–13], anti cancer peptide delivery and cell imaging studies [14,10,15]. Therefore, significant research effort has been devoted to the size control methods during CaP nanoparticle synthesis. The synthesis techniques include the solid state reaction [16], sol–gel [17,18], hydrothermal and emulsion techniques [19]. However, the common wet chemical precipitation method is considered the most convenient way to prepare CaP nanoparticles. In medical science, different types of bio polymeric materials are widely applied based on biocompatibility, non-toxicity and biodegradability [20]. Cyclodextrins are cyclic oligosaccharide, consisting of six, seven or eight α-D-glucose units covalently connected by α-(1.4) linkage to form torus shaped structure and are denominated as α-, β- and γ-cyclodextrin, respectively. The outer surface of cyclodextrin is hydrophilic due to the hydroxyl groups. They can form inclusion complexes with wide variety of guest molecules in their cavity through host–guest interactions and serve as model guest sites [21]. Newcastle disease virus (NDV) is an avian virus that causes disease in more than 250 avian species. In the poultry industry, it causes major economic losses. NDV, also named as avian *Paramyxovirus type 1* (APMV-1) comes under the family of *Paramyxoviridae* and genus *Avula virus*. Infection of NDV is mainly based two glycosylated proteins; the haemagglutinin-neuraminidase (HN) and Fusion (F) proteins. The

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http://dx.doi.org/10.1016/j.colsurfb.2013.12.017

penetration of NDV is mainly based on endocytosis process. Initially the virus attaches to the target cells through HN and sialic acid-containing receptors. After adsorption, NDV under goes conformational changes, trigger F protein cleavage in to F1 and F2 that determines the virulence of the virus [22–24].

In this work, the CaP nanoparticles were synthesized based on new continuous flow method using β-cyclodextrin as a biopolymer. These nanoparticles surface was modified with amino propyl triethoxysilane (APTES), and it offered amine functional group for NDV coupling. The NDV coupled nanoparticles were confirmed and characterized based on Scanning/transmission electron microscopy, Fourier transformed infrared spectroscopy (FTIR), X-ray diffraction patterns (XRD), Raman spectroscopy, differential scanning calorimetry (DSC) and energy-dispersive X-ray (EDX) spectroscopy. The immune response studies were carried out using chicken and their immunological responses were assessed in comparison to the conventional vaccine.

2. Materials and methods

2.1. Synthesis and characterization of nanoparticles

The β-CD–CaP nanoparticles were synthesized using a continuous flow process using solution A containing β-cyclodextrin, sodium hydroxide in milli-*Q*-water and it was pumped to the mixing tank at the flow rate of 5 mL/min. For CaP nanoparticles synthesis, solution B containing calcium chloride, and sodium dihydrogen phosphate dehydrate was pumped and mixed at a flow rate of 5 mL/min. After 2 h of continuous mixing, a white precipitate was formed in the mixing tank. Finally the synthesized particles were collected by laboratory filter press and it was dried at 55 °C hot air oven for 12 h.

For NDV coupling, the β-CD–CaP nanoparticles surface was modified with 3-aminopropyl triethoxysilane (APTES). After that 100 mg/mL of APTES modified nanoparticles, 5 mL of infective allantoic fluid was taken, and mixed for 3 h at room temperature. Finally, NDV coupled β-CD–CaP (β-CD–CaP-NDV) nanoparticles were collected by centrifugation at 8500 rpm for 5 min. The supernatant was collected to estimate the protein/vaccine encapsulation efficiency by using bicinchonic acid (BCA) protein assay.

2.2. Propagation and titration with NDV

Ten-day-old embryonated chickens eggs (ECE) from unvaccinated flocks were procured from Poultry Research Station (PRS), Chennai and used for NDV propagation. They were randomly checked for the presence of haemagglutination inhibition (HI) antibodies against NDV in their yolk samples and were found to be negative. The titration of NDV was done based on previous publication [25]. Briefly, the infectivity titre of NDV was determined by inoculating serial 10-fold dilutions (10^{-1} to 10^{-13}) of virus in the form of allantoic fluids into ECE. The end point titre was expressed as 50% embryo infective dose (EID_{50}) per mL as calculated by method of Reed and Muench. The haemagglutination (HA) and the HI tests were performed following the procedures specified in the Terrestrial Manual of the Office International des Epizooties. Briefly, the serum to be tested (25 μL) is diluted serially in two-fold dilutions across a 96-well micro titre V bottom plate. Then 25 μL of NDV containing 4 HA units is added to each well and allowed to incubate for 20 min. This is followed by the addition of 50 μL of 1% chicken red blood cells (RBCs). Appropriate controls to ensure that only 4 HA units of virus was used. The presence of antibodies against NDV virus would inhibit HA of the virus leading to settling of the RBCs to the bottom of the well resulting in a button. If no antibodies are present, the virus added would cause HA leading to 'pellicle' formation. Infective allantoic fluid containing NDV having HA titre of 2^{11} and EID_{50} $10^{10.45}$ was used for coupling with β-CD–CaP particles.

2.3. Whole blood haemolysis assay

Chicken blood was collected in vacutainers (Becton Dickinson, Inc., USA) containing 24 IU sodium heparin. A volume of 200 μL uncoagulated whole blood was taken in microfuge tubes separately, and β-CD–CaP nanoparticles at varying concentrations were added. After that, the samples were gently mixed in a rotary shaker, and then incubated for 4 h at 37 °C. The samples were then centrifuged at 1200 rpm for 10 min to collect the plasma. The plasma was further centrifuged at 13,000 rpm for 15 min to remove the nanoparticles and the supernatant was analyzed for the presence of the haemoglobin by specific 545 nm spectrophotometric absorption. A 2% Triton-X 100 was used as positive control and normal saline as the negative control.

2.4. Red blood cell haemolysis assay

Chicken RBC was separated from the chicken blood by centrifugation. From that 200 μL of RBC was added to β-CD–CaP nanoparticles at varying concentrations. The mixture was incubated for 2 h at 37 °C, centrifuged at 1500 rpm for 5 min and the supernatant was checked for released haemoglobin at 545 nm. A 2% Triton-X100 was used as positive control and normal saline as the negative control. The percentage of haemolysis was calculated using a relative method based on the optical density (OD) values as follows:

$$\%\ \text{of haemolysis} = \frac{(\text{OD sample} - \text{OD negative})}{(\text{OD positive} - \text{OD negative})} \times 100.$$

2.5. Red blood cells aggregation tests

Chicken RBC collected as above was mixed with β-Cd–CaP nanoparticles at varying concentrations and incubated for 30 min at 37 °C. Aggregation of the RBC was observed in phase contrast microscope at a magnification of 40×.

2.6. Cytotoxicity studies

For this study, 10 mL of chicken blood was collected from unimmunized white leghorn chickens at the age of 4 weeks. A peripheral blood mononucleated cell (PBMC) were separated using Ficoll-Paque (Amersham Pharmacia Biotech, USA) and washed twice in cold Roswell Park Memorial Institute (RPMI) 1640 (Gibco). PBMCs were seeded at a density of 10^6 cells/mL on 24-well cell culture plates (Nunc, USA) @ 1 mL per well. All cells were cultured in a complete RPMI 1640 medium containing 10% heat inactivated fetal bovine serum (FBS) (Gibco, USA) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) (Gibco). The cells were incubated at 41 °C in 5% CO_2 for 24 h following the addition of 10 to 150 mg/mL of nanoparticles. Following incubation, the growth media were replaced with fresh medium (0.1 mL of medium and 0.1 mL of MTT reagent) and the cells were incubated for 4 h. After that, the media were removed and dimethylsulfoxide (0.3 mL) was added into each well to dissolve the internalized purple formazan crystals and the samples were assayed by at 550 nm absorption. The background absorbance was measured at 690 nm and subtracted from the 550 nm measurement. The percentage (%) of cell viability was calculated by dividing the optical density values (OD) of the treated groups (S) by the OD of the controls (C) ([S/C × 100%]).