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In ovo delivery of Newcastle disease virus conjugated hybrid calcium phosphate nanoparticle and to study the cytokine profile induction

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ABSTRACT

In this report, the hybrid calcium phosphate (CaP) nanoparticles were synthesized and functionalized with Newcastle disease virus (NDV). These nanoparticles were synthesized by a combination of co-precipitation and polymerization process and functionalized with amino propyl triethoxy silane before coupling to NDV. The 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide (MTT) assay of chicken spleen cells incubated with these nanoparticles indicated that, these particles did not exert any significant cytotoxicity. The effects of hybrid CaP nanoparticles on cell cycle were assayed using a flow cytometer. The results demonstrated that the cell viability and proliferation capacity of spleen cells were not affected by hybrid CaP nanoparticles compared with their control cells. The hybrid CaP nanoparticles were characterized by scanning/transmission electron microscopy (SEM/TEM); Fourier transformed infrared spectroscopy (FTIR), X-ray diffraction patterns (XRD), Raman spectroscopy and energy-dispersive X-ray spectroscopy (EDX). These methods revealed that NDV was successfully conjugated on nanoparticles. The ability of the hybrid CaP nanoparticles to induce different cytokine mRNAs in the spleen cells of 18-day old embryonated chicken eggs (ECEs) was studied by quantitative real time polymerase chain reaction (qRT-PCR). NDV conjugated particles induced a high expression of Th1 cytokines such as interferon (IFN)-α, tumor necrosis factor (TNF)-α of and Th2 cytokines, interleukin (IL) 6 and IL-10. Uncoupled NDV induced only Th1 cytokines, IFN-α, INF-γ and TNF-α. The hybrid particles alone did not induce any cytokines. This confirmed that nanoparticle coupling could induce differential cytokine profiles and hence can be used as an alternate strategy to direct favorable immune responses in animals or chickens using appropriate vaccination carrier.

1. Introduction

Calcium phosphate (CaP) nanoparticles have become the nanoparticle of choice for *in vivo* applications due to its biocompatibility [1]. The World Health Organization has approved CaP nanoparticles as a safe delivery material for biological applications. In recent years, hybrid nanomaterials have wider biomedical applications because of the potential to combine organic and inorganic molecules. These have applications (i) in biomedical device fabrications, (ii) as a carrier for drug delivery system, (iii) as a carrier for cell immobilization studies, (iv) as a carrier for signaling molecules and (v) in bioseparation membrane designing [2–5]. At the laboratory level, the nanoprecipitation method is widely used for hybrid particle preparations [6]. In this approach two miscible solvents are used for the preparation and the particle size is mainly dependent on polymer concentration, ratio of the two solvents and mixing rate.

Poly N-vinyl pyrrolidone (PVP) and poly(ethylene glycol) (PEG) are the most widely used water soluble polymers in biomedical and pharmaceutical applications due to its non-toxic, non-carcinogenic nature and potential for easy attachment with nanoparticles or to other solid matrices [7–10]. During hybrid particle synthesis, PEG maintains the stability of the hybrid network and reduces nanoparticle aggregation. Further it also offers functional groups for modification of the hybrid nanoparticle surface targeting ligands for cell or tissue specific drug or vaccine delivery. PVP is mostly used as a stabilizer for nanoparticle preparations. It also has a high dielectric strength but solubility is limited in water and alcohols [11,12]. Free PVP is very hydrophilic because of the polar pyrrolidone functionality attached to the main alkyl chain. It dissolves in water, ethanol, and chloroform, while PVP-capped nanoparticles are poorly soluble in water and to overcome this problem we have developed hybrid network between PVP and PEG.

In certain context, chick embryo based experimental studies are preferred rather than use of human cell lines due to their low cost and simplicity. Apart from this, the embryo in the egg is easily accessible; transplants are not rejected because their immune system is not mature completely and legal and ethical restrictions are limited. Previous studies indicated that, the chicken embryos are highly-organized living

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structures and serve as better models for estimating the effects of nanoparticles on intact organism than isolated *in vitro* cell cultures [13–15]. In addition to antibodies, cytokines are major mediators of immune responses and their types and levels determine the outcome of infections and vaccinal efficacy [16–18].

Recent studies have shown that merging properties of individual materials (inorganic/organic materials) in single networks offered multiple functionalities. However, most of the reported syntheses of hybrid particles have involved expensive, toxic reagents and complicated multistep syntheses. However, in this work we developed a physical mixing procedure for synthesis at a suitable temperature without using any initiators. This approach was more convenient, cheaper and less time consuming. Apart from this, the pyrrolidone ring of PVP binds easily with PEG molecules. The CaP core was developed by co-precipitation process using β-cyclodextrin as template, and it showed very high affinity towards the hybrid network. After that, the surface was modified with aminopropyl triethoxy silane (APTES). The ligands offered free amino functional group for Newcastle disease virus (NDV) binding. The main aim of this study was to functionalize hybrid nanoparticles with NDV and study the induction of cytokines in the spleen of embryonated chicken eggs (ECEs). The NDV conjugated hybrid shell coated CaP nanoparticles induced differential cytokine release as compared with NDV free solutions.

2. Materials and methods

2.1. Preparation and characterization of nanoparticles

For the preparation of CaP particles, solution A was prepared by mixing 0.7 g of β-cyclodextrin and 10 mg of sodium hydroxide in 30 mL milli-Q-water. After that solution B was prepared by using 0.7 g of calcium L-lactate as a calcium source and 0.5 g of sodium dihydrogen phosphate as a phosphate source and mixed with 30 mL of milli-Q-water. For the co-precipitation process, Solution A and solution B were mixed together under continuous stirring, and then centrifuged at 6500 rpm.

The hybrid network was formed by using PVP and PEG. For the preparations, 2 g of PVP and 2 g of PEG were mixed with 80 mL of deionized-water and 20 mL of ethanol. Finally the solution was heated at 80 °C for 30 min for cross-linking and allowed to cool at room temperature (RT). For hybrid nanoparticles, the formed CaP pellet was mixed with milli-Q-water and hybrid network and stirred overnight at RT. For the amino group insertions, the particles were mixed with APTES and stirred for 12 h, after which the particles were collected by using centrifugation at 6000 rpm.

The nanoparticle's morphology, chemical composition, crystalline structure and thermal properties were examined by using TEM (S-3400N model, Hitachi), SEM–EDX (Quanta 200 FEG), FTIR (PerkinElmer Spectrum 1 FTIR instrument), Raman spectra (Bruker RFS 27: Stand-alone FT-Raman spectrometer), and XRD (Bruker) at the Sophisticated Analytical Instrumentation Facility (SAIF), Indian Institute of Technology, Chennai using established methods.

2.2. Quantification of amine groups

Quantification of primary and secondary amines on the nanoparticle surface was carried out by ninhydrin colorimetric assay [19]. The ninhydrin reagent (500 μL of 0.2% w/v in 0.1 M buffer phosphate, pH 9) was added to 200 μL of nanoparticle sample and the mixture heated in a boiling water bath for 15 min. Samples were cooled to room temperature and placed on a magnetic separator to remove the nanoparticles. The absorbance of supernatants was measured at 570 nm on a microplate and the amine content quantified using APTES standard curves.

2.3. Cytotoxicity studies

For this study, initially spleens were collected from unimmunized 4 week old specific-pathogen-free (SPF) white leghorn (WL) chickens. Leukocyte suspension was prepared from the spleens by slow speed density gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, USA) and washed twice in cold Roswell Park Memorial Institute (RPMI) 1640 (Gibco). Splenic macrophages were seeded at a density of 10^6 cells/cm^2 in 24-well cell culture plates (Nunc, USA). All cells were cultured in complete RPMI 1640 medium containing 10% heat inactivated fetal bovine serum (FBS) (Gibco, USA), 5% chicken serum (Gibco), and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) (Gibco). After 2 h, nonadherent cells were removed by gently washing the 24-well plates three times with warm complete RPMI 1640 medium 37 °C in a 5% CO_2 incubator. The cell cytotoxicity effects of the nanoparticles were tested by using spleen cells and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay protocol [20]. The cells were seeded into 96-well tissue culture plates at a density of 50,000 cells per well and cultured for 24 h with minimum essential medium (MEM). 1 to 5 mg/mL of nanoparticles + culture medium was prepared separately, after 24 h, the original medium was replaced with the medium containing the nanoparticles. This was incubated for 24 h and growth media were replaced with fresh medium (0.1 mL of medium and 0.1 mL of MTT reagent) and again cells were incubated for 4 h. Media was removed and dimethylsulfoxide (0.3 mL) was added into each well to dissolve the internalized purple formazan crystals. The samples were assayed using 96 well plates 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%]).

Table 1
Oligonucleotide primers used for the qRT-PCR of chicken cytokine genes.

Gene	Seq 5′–3′	Position	Product (bp)	Accession no.
Beta actin FP	GTACCCTGGCATTGCTGAC	983–1001	182	NM-205518
Beta actin RP	CGGATTCATCGTACTCCTGC	1145–1164		
IFNα FP	GTCTTGCTCCTTCAACGACA	198–217	303	GU119896
IFNα RP	GCGCTGTAATCGTTGTCTTG	481–500		
IFNγ FP	CCAAGAAGATGACTTGCCAGA	98–118	349	NM-205149
IFNγ RP	ACCTTCTTCACGCCATCAGG	427–446		
TNFα FP	CTTCTGAGGCATTTGGAAGC	39–58	351	AY765397
TNFα RP	ACTGGGCGGTCATAGAACAG	370–389		
IL2 FP	TTGGCTGTATTTCGGTAG CA	172–191	169	AJ578467
IL2 RP	GTGCACTCCTGGGTCTCAGT	265–284		
IL4 FP	GGAGAGCATCCGGATAGTGA	99–118	186	NM-001007079
IL4 RP	TGACGCATGTTGAGGAAGAG	265–284		
IL10 FP	GCTGCGCTTCTACACAGATG	296–315	203	EF554720
IL10 RP	TCCCGTTCTCATCCATCTTC	479–498		