Evaluation of Nanoencapsulated DNA Vaccine for RSV

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ABSTRACT

In the present study, antigenic regions of RSV F, M2 and G genes were cloned into phCMV1 vector resulting in development of a DNA vaccine vector named DR-FM2G. The DNA vaccine vector was used to formulate DNA-nanoparticles complex using chitosan by a complex coacervation process. Stability of nanoparticles was investigated at different pH values. The effect of DNA concentration on release rate from chitosan nanoparticles was examined using similar intestinal fluid (SIF) and similar gastro fluid (SGF). Cytotoxicity of chitosan nanoparticles was measured by MTT dye reduction assay, and nanoparticles were evaluated by SEM and TEM. DRFM2G vector and DNA+nanoparticles were used to vaccinate BALB/c mice.

Keywords; Respiratory syncytical virus, DNA vaccine, Chitosan nanoparticles

INTRODUCTION

Human Respiratory Synctical virus (RSV) is a major respiratory tract pathogen causing pneumonia and death in children and the elderly. The RSV genome encodes 11 proteins, out of which the fusion (F), attachment (G) glycoprotein and matrix protein (M2) proteins are targeted for vaccine development(Ref 1).
However, the delivery of naked DNA to mucosal surfaces normally produce little or no transfection of epithelial cells and poor immunological responses. A suitable delivery system is required that will lead to an improved presentation of DNA to antigen presenting cells. Naked DNA is a large, charged molecule, that has little tendency to be absorbed across mucosal surfaces. The addition of oppositely charged lipids and polymers, leads to the self-assembly of the DNA to form nanoparticles. Cationic polymeric materials are used as vehicles because of the ease of complex formation and high stability. Chitosan is a preffere cationic polymer for gene delivery systems due to its low toxicity and biocompatibility. It can condense DNA, which can ensure smaller diameters and easier entry into cells and nucleus. Moreover, DNA/chitosan nanoparticles could partially protect the encapsulated DNA from nuclease degradation(Ref 2,3).

MATERIALS&METHODS

Plasmid Preparation

RSV F-M2-G gene was amplified using the pet32 FM2G plasmid as a template. The primers used in the PCR reaction added EcoRI and BamHI restriction sites to allow restriction cloning of the F-M2-G gene into the phCMV1 vector. Purified FM2G gene and purified phCMV1 were then digested with EcoRI and BamHI restriction enzymes. Next, the FM2G gene was cloned into the pHCMV1 vector using T4 DNA ligase. Restriction analysis and sequencing were used to verify the presence and orientation of the FM2G gene insert.

Preparation of chitosan-DNA nanoparticles

A chitosan solution (4mg/ml in 5 mM sodium acetate buffer, pH 5.5) and a DNA solution of 0.2 mg/ml in 45mM sodium sulfate solution were preheated to 55° C separately. The ratio of DNA/ chitosan in all formulations was kept at 5:1 by weight. Both solutions were quickly mixed together and vortexed for 15-30 s (Ref. 3).

Evaluation of encapsulation efficiency and DNA loading level

Nanoparticle suspensions were centrifuged in a series of 35-55-85% (w/v) sucrose gradients at 40.000xg for 20 min.The supernatant was analyzed for DNA concentrations, which accounted for the non-trapped DNA, using a nano-drop spectrophotometer. The concentration of chitosan in the supernatant was measured by the ninhydrin assay, from which the amount of chitosan entrapped in the nanoparticles was calculated. DNA loading level in nanoparticles was calculated based on the amount of DNA and chitosan in the nanoparticles.

In vitro release studies

Release of plasmids from chitosan nanoparticles was determined in SGF (0.55 M glycine-HCI buffer 0.5%pepsin, pH 2.0) and SIF (0.067 M phosphoric acid buffer 1.0% trypsin, pH 7.0) at (37$\pm$ 0.5) ºC with mild shaking and at appropriate time intervals (15 min, 1h,5h, day 1,2,3,4,5,7).Samples were taken and supernatants were separated by centrifugation. The released DNA was measured spectrophotometrically at 260 nm.

Cytotoxicity assay

Cytotoxicity of chitosan and chitosan-DNA nanoparticles was measured using the MTT dye reduction assay in Cos 7 and HEp2 cells. Cells were seeded in a 96-well plate at a density of $2.0x10^4$ cells/well and incubated overnight at 37 °C. Then the cells were incubated in 100 µl serum free medium containing selected amounts (from 50 to 600ug/ml) of chitosan and chitosan-DNA nanoparticles. After 24 and 48 h, the medium was removed and the cells were rinsed twice with sterile PBS. Next, 10ul of MTT (5mg/ml) solution was added into each well and allowed to react for 4 h at 37 °C. DMSO (150 µl) was added to each well and the plate was incubated for 30 min at room temperature. Absorbance at 490 nm was measured with an ELISA plate reader.

Stability tests

Investigation of particle disintegration caused by pH changes were performed by adjusting pH with 1M HCI,1M NaOH, or addition of sodium acetate buffer (1M,pH 5.5). After 30min, 1h and 2 h of incubation in an Eppendorf shaker at 37°C and at 400rpm, the samples were analyzed by agarose gel electrophoresis with 0.8% agarose in 1xTAE –buffer.

Temperature stability experiments werecarried out using Differential scanning calorimetry (DSC). DSC experiments were done with a Mettler Toledo DSC 822^e instrument at a sample heating rate of 5°C /min in the temperature range of 30 to 300°C under nitrogen atmosphere and then cooled down from 300 to 30 °C at the same rate.

In vitro transfection

Transfection of DRFM2G clone into Cos 7 cells (180000cells/well) and HEp2 cells (2000000cells/well) were done using Exgene 500. Cells were seeded into 8-well-plates and incubated for 24h at 37 °C with 5% CO_2 before transfection in Minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 75 U/ ml Penicillin, 100 µg/ml Kanamycin and 75 µg/ml Streptomycin.

Transfection with Chitosan/DRFM2G DNA nanoparticles

Cos7 cells and HEp2 cells in monolayer, grown in 8 chambered slides, were transfected with DRFM2G DNA and different concentrations of Chitosan/DRFM2G DNA nanoparticles after 24 h incubation in MEM 10. After 48h incubation with nanoparticles and DNA, cells were washed with phosphate buffer saline (PBS) and fixed using 10% trichloroacetic acid for 15 min. Cells were then successively washed in 70%, 90%, and 100% ethanol for 5 min. each. After a subsequent wash with PBS, the cells were incubated in blocking buffer (3% dry milk in PBS) for 30 min followed by 3 washes with PBS. The fixed cells were incubated for 1 h at room temperature with monoclonal mouse antibody to RSV F in antibody buffer (2% dry milk in PBS). The cells were washed three times for 5 min each in PBS and then incubated for 1 h at room temperature with FITC-conjugated goat anti-mouse IgG (H+L) secondary antibody in antibody buffer. Non-specific binding was eliminated by three washes in PBS. Subsequently, the nuclei of the cells were stained using DAPI, and visualized with Nikon (Model 1X51) fluorescent microscope.

Morphology

Transmission Electron Microscopy (TEM) analysis

High resolution TEM (HRTEM) was performed using a JOEL-2010 machine. HRTEM samples were prepared by dispersion of Chitosan-DRFM2G DNA nanoparticles in ethanol and drop of the solution was placed on a copper grid (carbon coated copper grid-200mesh), dried in air and used for HRTEM analysis.

Scanning Electron Microscopy (SEM) analysis

Morphological analysis was carried out using JEOL JSM 5800 SEM. The samples were placed on a double sided carbon tape and coated with gold/palladium to prevent charge buildup in the specimen by electron absorbtion .

Mice and Immunization

Four groups (10 each) 4-6 wk old BALB/c female mice were immunized with four immunogens (DRFM2G, DNA, nanoparticles, RSV and PBS). Animals were immunized (IM) thrice with 50 µg of purified DNA in 50 µl . Others were immunized intranasally thrice with 50ug of nanoparticles on day 1, 15, and 29, or PBS. The fourth group were immunized with live RSV long strain only once on day 1. Sera and saliva samples were collected on day 0, 14, 28, and 49. These samples were pooled into groups of at least 3 mice each and stored at -80oC for antibody response and isotyping analysis. Vaccinated mice were challenged with live RSV at day 55 to assess protection capabilities of the DNA vaccine vector.

Statistical analysis

The data are presented as means and standard deviations; differences were analyzed using the two tailed Students's *t-test* with P∠ 0.05 as the level of significance.

RESULTS

Chitosan/DNA complexation is known to be influenced by many factors such as salt concentration, pH, polymer charge density, and polymer molecular weight, polymer to DNA ratio, polymer tertiary structure and polymer deacetylation degree (Ref 3). For the complex coacervation method, the first step of chitosan-DNA nanoparticle formulation was the complex formation between the two opposite charged polyelectrolytes, chitosan and DNA. Sodium sulfate was included as a desolvating reagent to facilitate the complexation (Ref 3) reported that, with a formulation process at pH 5-5.8 and with a solution temperature above 50 °C. In our studies, the temperature was set at 55 °C, and pH to 5.5 in the presence of 45mM sodium sulfate. Encapsulation efficency of the nanoparticle was 94%.

TEM and SEM analysis

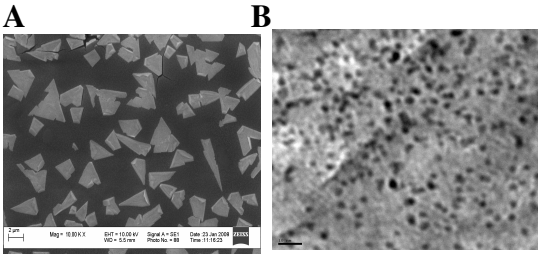

Figure 1: SEM (A) and TEM(B) images of chitosan/DNA nanoparticels (arrows, bar = 2nm)

DNA release from chitosan/DNA nanoparticles

When nanoparticles were incubated with SIF, after 10% release occurred in the first h, 35% after 24 h, and 60% after 7 days. When incubated with SGF, 25% release occurred in the first h, 30% after 24 h, and 35% after 7 days (Figure 2).

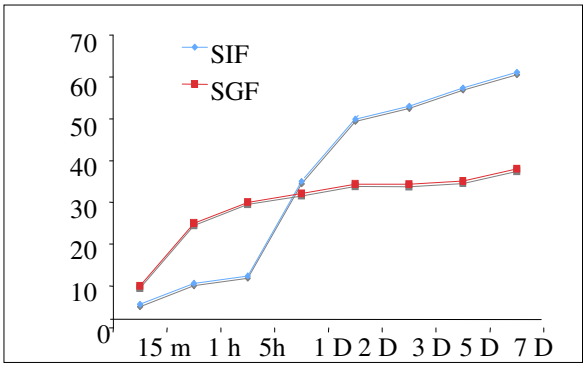

Figure 2: The release of DNA from chitosan nanoparticles (m, h, D refers to minute,hour and day respectively, x and y value refers to time and release Ration(%) respectively)

Cytotoxicy Assay

The cytotoxity of chitosan increased as concentration increased and most cells were dead at 400µg/ml (Fig 3). Chitosan/DNA nanoparticles were significantly less cytotoxic compared with chitosan in both cell lines. Cells grew well even at 200µg/ml.

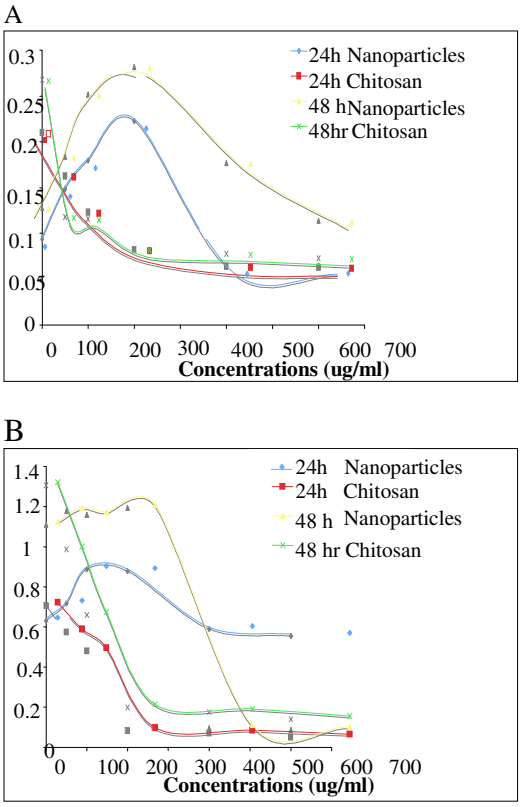

Figure 3: MTT assay for cytotoxicity of chitosan and chitosan/DNA nanoparticles in Cos 7 cell lines (A) Hep2 cell lines(B)

Stability Studies

Chitosan nanoparticles released only 21-25% of DNA in 2 h at pH 10. DSC results showed that chitosan nanoparticles were stable until 80°C.

Transfection studies

Fluorescence imaging showed that within 24h of transfection with Chitosan/DRFM2G DNA nanoparticles, cells were able to express GFP (Fig4), showing the protective effect of chitosan for DNA transport into the cytosol.

CONCLUSION

The described method is suitable to prepare cationic nanoparticles capable of effectively complexing DRFM2G-DNA. The main advantage seems to be the enhanced transfection efficiency. Moreover, chitosan nanoparticles were found to improve cell viability.

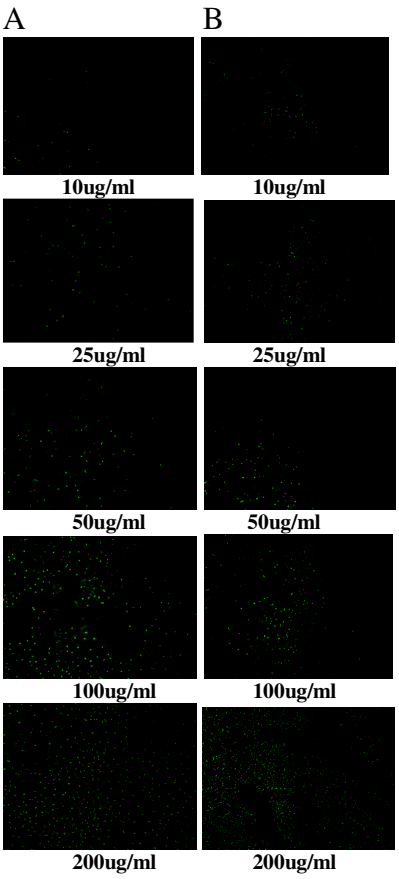

Figure 4: Expression of green fluorescence protein in A) Cos 7 cells and B) Hep 2 cells after 24 h transfection with nanoparticles. Pictures were taken after 48 h postransfection under fluorescence microscope**.**

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