Development of PHEMA-Chitosan Nano-spheres and Encapsulation of RSV-F DNA Vaccine into these Nano-carriers

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ABSTRACT

Respiratory Syncytial Virus (RSV) is a negative sense RNA virus that causes severe and long lasting pulmonary infectious in infants. Unfortunately, an effective vaccine preventing re-infection of RSV has not been developed. Poly hydroxyethyl methacrylate (PHEMA) nano-spheres coated by chitosan is a promising vaccine delivery vehicle. In this study, RSV full-length fusion (RSV-F) gene was constructed into phCMV1 vector. The *in vitro* expression of RSV-F protein was verified by immunofluorescence microscopy and flow cytometry. PC composite nano-spheres were synthesized as a nano-scale DNA vaccine carrier. The size and the shape of PHEMA-Chitosan (PC) nanospheres were verified using TEM. The size of PC nano-spheres was in a range of 100-150 nm. The Cos-7 cells were 85% viable up to 2.5% PC. To our knowledge, this is the first study encapsulating a DNA vaccine into PC with high efficiency. The aim of the current study is to develop an effective DNA vaccine using nanomaterials.

Keywords: rsv-f, dna-vaccine, phema-chitosan, nanoparticles, encapsulation

1 INTRODUCTION

Respiratory Syncytial Virus (RSV), a negative sense mRNA virus, is a member of the genus *Pneumovirus* classified under the family *Paramyxoviridae*. RSV is a common viral pathogen that causes serious lower respiratory tract diseases, bronchiolitis and pneumonia, especially among infants, young children, elderly and immunocompromised people [1]. There is only one commercial antiviral drug (ribavirin, a purine nucleoside analogue) available in the market to cure RSV infections. An effective vaccine conferring long lasting immunity and protection prior to infection could be a better solution rather than an expensive drug showing variable efficiency [2]. Despite the high disease frequency and clinical importance of RSV infections, none of the vaccine has displayed any significant improvement for decades [1, 3]. Antigenic variations of RSV could have played a role in re-infection of RSV disease. RSV has 10 genes encoding 11 viral proteins [3], among which RSV attachment (RSV-G) and fusion (RSV-F) proteins, leading to viral attachment and fusion to the host, are located on the outer surface of the virus. It is thought that surface proteins directly interact with immune cells and result in stimulation of the host immunity. The RSV-G gene is very diverse, whereas RSV-F gene is highly conserved among the different strains of RSV [3, 4]. Thus, the rationale of our study was the development of an RSV-F DNA vaccine that could confer long lasting immunity against different strains of RSV.

The most common issue, which needs to be addressed in case of either protein or DNA vaccines, is their low bioavailability in living systems owing to their degradation and instability due to the bio-barriers or low permeability through the mucosal membranes. Limited capabilities of recombinant vaccines can be improved by incorporating into nano-structures that protect them from biological barriers and provide controlled delivery *in vivo* [5, 6]. pHEMA nano-spheres coated by chitosan could be one of the most promising vaccine adjuvants to improve delivery of either DNA or protein vaccines. The properties of PC nanoparticles of being biologically safe, biodegradable and inexpensive make them unique in comparison to other nanomaterials [7]. Positively charged chitosan having sialic groups of mucin can electrostatically bind to epithelial cells on mucosal surface of respiratory tract and can penetrate into the deep tissues and cells due to its nano-scale size [5]. pHEMA is one of the currently used synthetic nanomaterials approved as biologically safe by FDA (Food and Drug Administration) in USA for industrial, biomedical and pharmaceutical applications. The abundance of hydroxyl groups in its structure makes attachment possible to any biomolecules such as DNA or protein. The aim of this study was to synthesize pHEMA-Chitosan nano-spheres as drug delivery system and encapsulation of RSV-F DNA vaccine into these nano-carriers to enhance the potential efficiency of the DNA vaccine.

2 MATERIAL and METHODS

2.2 Construction of Recombinant RSV-F DNA Vaccine Plasmid and Insertion of GFP Reporter Gene

RSV-F DNA sequence acquired from Collins et al. (1984) [8] was synthesized by Epoch labs (Texas, USA). RSV-F DNA sequence was amplified by polymeric chain

reaction (PCR) by using RSV-F specific primers. The size of the PCR product was confirmed on 1% agarose gel. PCR-amplified DNA was cleaned by phenol-chloroform-alcohol (PCA) precipitation and the precipitate was dissolved in nuclease free water. Both RSV-F fragment and phCMV1 DNA vector were digested with *BamHI* and *NotI* restriction enzymes (RE) in water bath at 37^{o}C for 2 hours. The digested DNA pieces were separated on 1% low melting agarose gel prior to DNA isolation by QIAGEN gel extraction kit (QIAGEN, Valencia, California). The purified DNA pieces were sealed by T4 DNA ligase enzyme by incubating overnight at 16^{o}C. The ligation mix was transformed into the competent cells of *Escherichia coli* DH5α strain. The competent cells were spread on kanamycin (50 µg/ml) supplemented Lauria Bertani (LB) agar and clones were grown overnight in kanamycin supplemented LB broth. Positive clones were verified by *BamHI* and *NotI* RE digestion, observation of the DNA pieces on 1% agarose gel and DNA sequencing as well.

A reporter gene, green fluorescence protein (GFP), was inserted between *NotI* RE cut sites of phCMV1 vector and RSV-F gene. *NotI* RE cutting sites were introduced into both sides of GFP gene by PCR before constructing into the vector and the clone was named as PFG. The recombinant PFG vector was purified by using the QIAGEN Endofree Giga kit and the purified PFG DNA aliquots with the concentration of 1 mg/ml DNA were kept at -80^{o}C until further use for *in vitro* transfection, encapsulation into NPs and *in vivo* expression studies.

2.3 *In vitro* Expression of RSV-F-GFP Protein

To show *in vitro* expression and efficiency of phCMV-F-GFP DNA vaccine construct, PFG DNA was transfected into Cos-7 cells using electroporation technique. Cos-7 cells were grown in minimal essential medium up to 90 % confluency and cells were collected by trypsinization with TrypLE^{TM} (Life Technologies Inc.). The cell pellet containing 1x10^{6} cells/reaction was suspended in suspension buffer composed of 82 µl of cell line nucleofactor solution R and 18 µl of supplement-1 prior to addition of 10 µg DNA (10 µl of 1µg/µl stock DNA). DNA and cell solution were mixed gently and immediately exposed to electroporation (Amaxa Nucleofector II Lonza, Switzerland) in cuvette. Subsequently, 500 µl of cell medium was added into the electroporated cell solution, all content of the cuvette was transferred into a 6 well plate, final media volume was made up to 2 ml per well and cells were incubated at 37^{o}C, with 5% CO_2 for 3 days. At the end of the incubation time, plates were observed under immunoflourescence microscope (Nikon ECLIPSE Ti, Nikon Instruments, Melville, New York, USA). The cells were then collected by trypsinization before they were run on a Beckton Dickinson FACS CantoII flow cytometry (BD Bioscience, San Jose, USA). phCMV1 vector was used as a negative control whereas phCMV1-GFP construct was used as positive control in the same experiment.

2.4 Purification of Chitosan and Synthesis of Chitosan-pHEMA Composite Nanoparticles

The protocol for the purification of chitosan nanoparticles was adapted from Gan et al. (2005) [9]. Briefly, five grams chitosan (MMW) was dissolved in 70 ml of 1M sodium hydroxide (NaOH). Following centrifugation, pelleted chitosan was washed twice and supernatant was removed. Thereafter, chitosan pellet was dried overnight at 40^{o}C in an incubator. Next day, the dried chitosan was completely dissolved in 0.1M acetic acid. The pH of solution was adjusted to 8.0 with 1M NaOH until pure chitosan appeared as white precipitate. Precipitated chitosan was washed twice and dried. Final pellet was kept at room temperature until use.

75 mg of purified MMW chitosan was dissolve in 10 ml 0.2M Nitric acid solution by vigorous stirring at 40^{o}C until chitosan totally dissolved and then chitosan solution was filtered to remove insoluble particles. Complex coecervative method was modified from Atyabi et al (2008) [7] and followed to prepare pHEMA-Chitosan nanoparticles (PCNPs). Clear chitosan nanoparticle solution was stirred at 40^{o}C for additional 10 minutes and ammonium cerium (IV) nitrate (CAN) (0.08 M) and HEMA (0.16M) was slowly added into 10 ml chitosan nanoparticle solution under continuous stirring. The nanoparticle preparation was continued under gentle stirring at 40^{o}C for next 40 minutes. The temperature of solution was dropped down to room temperature before adjusting the final pH to ~4.5 with 5 M NaOH.

2.5 Encapsulation of PFG DNA into Chitosan-pHEMA Composite Nanoparticles

Same nanoparticle preparation method was followed during DNA encapsulation into pHEMA-Chitosan nanoparticles. DNA was simply mixed with HEMA in an eppendorf tube before addition into chitosan nanoparticle solution. Then HEMA-DNA mixture was added into Chitosan+CAN solution drop by drop with continuous stirring. Final DNA concentration was adjusted to 100 µg/ml in PCNPs-DNA solution. All PCNPs or PCNPs-DNA solutions were stored at pH ~4.5 in refrigerator until use.

2.6 Transmission Electron Microcopy (TEM)

Transmission electron microscopy (TEM) (Zeiss, Gottingen, Germany) was used to observe the morphology and size of PCNPs and DNA encapsulated within PCNPs. Nanoparticle solutions were diluted in distilled water to 1/50 (v/v) before dispersion onto the carbon-coated copper grid (200 mesh). After 5 min incubation, samples were stained with 1% phosphotungstic acid. The morphology and size were verified by two independent experiments with multiple scans from randomly chosen area of TEM images.

2.7 *In vitro* DNA Release from PCNPs-DNA Complex

Considering the administration routes of PCNPs-DNA (intramuscular or intranasal routes), the release of phCMV1-F vector DNA from PCNPs complex was studied in PBS. One ml of PCNPs and PCNPs-DNA solutions were centrifuged at 14,000 rpm for 10 min. The supernatant was removed. Pellet was washed once in one ml of 1% PBS and centrifuged again. The supernatant was saved as time zero. Pellet was suspended into 1 ml of 1% PBS prior to incubation in shaker incubator at 37 ^{o}C, 200 rpm. Sample collection was performed by repeating the same steps; centrifugation followed by collection of supernatant and re-suspending the pellet in one ml of 1% PBS. Samples were collected at times, 0h-1h-2h-4h-8h and from day-1 to day 15. The amount of released DNA was measured using NanoDrop ND-100 (Thermo Fisher Scientific, Inc.) at 260 nm wavelength. The determined DNA concentrations were cumulated over the studied time period. The data was estimated as percentage release with time.

2.8 *In vitro* Cytotoxicity of NPs

MTT dye reduction assay (Promega) was performed to determine *in vitro* cytotoxicity of PCNPs and DNA encapsulated within PCNPs on Cos-7 cells. According to the protocol, Cos-7 cells when more than 90 % confluent, were harvested using TrypLE^{TM} and 1x10^{4} cells/well in 100 µl were seeded into each well of 96 well plate. Cells were allowed to grow overnight in incubator at 37^{o}C, 5% CO_2 humidified atmosphere until nanoparticles were added into each well. After overnight incubation, media from each well were replaced with the corresponding concentration of NPs-cell medium solution prepared by serially diluting nanoparticles from 5% to 0.1% (v/v) with cell culture medium supplemented with 10% FBS. Cos-7 cells treated with NPs were further incubated at 37^{o}C, 5% CO_2 for 24, 48 and 72 hours. At the end of the corresponding incubation time, 15 µl of MTT dye was added into each well and plate was allowed to incubate for next 4 hours in incubator in dark. The reaction was then stopped with addition of 100 µl of stop solution into each well. The absorbance of each plate was measured at 570 nm on a TECAN Sunrise enzyme-linked immunosorbent assay plate reader (TECAN, US Inc., Durham, North Carolina). Non-treated cells in the media were used as a growth control. Data was estimated as % cytotoxicity compared to control.

3 RESULTS and DISCUSSION

3.1 *In vitro* Expression of PFG in Cos-7 Cells

After 3 days incubation of cells, transfection was verified by green fluorescence using an immunoflourescence microscope and flow cytometry analysis. The green fluorescence corresponds to GFP production, which is tagged at the end of the RSV-F protein (see Fig. 1). The negative control (phCMV1) and positive control (phCMV1-GFP) were tested via flow cytometry analysis. Transfection process was very efficient, because expression of GFP-alone (positive control) was detected up to 90% with both flow cytometry and immunofluorescence imaging. On the other hand, no green color signal was detected with flow cytometer for negative control (phCMV1). The expression efficiency of RSV-F-GFP protein was defined around 15% by flow cytometry analysis. In conclusion, the *in vitro* expression of vaccine DNA, RSV-F-GFP protein, was shown in Cos-7 cells with both flow cytometry analysis and immunofluorescence imaging.

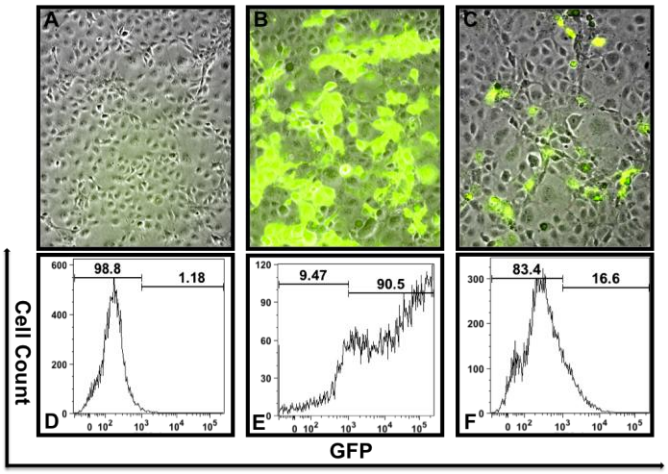

Figure 1: Immunofluorescence microscopy and flow cytometry analysis showing the *in vitro* expression of negative control-phCMV1 vector (A, D), positive control-GFP (B, E) and RSV-F protein (C, F), respectively.

3.2 Synthesis and Characterization of PCNPs

According to the TEM images, the diameter of the pHEMA spheres coated with a thin layer of chitosan was ranging from 100 nm to 150 nm. The size and shape of the composite nano-spheres was same even after encapsulation of DNA at the concentration of 100 µg/ml PCNPs solution (see Fig. 2). The chemical interaction and formation of bonds between chemical groups of HEMA and chitosan was shown with FTIR spectroscopy analysis. The zeta potential values of the PCNPs either alone or with DNA, were almost same and positively charged.

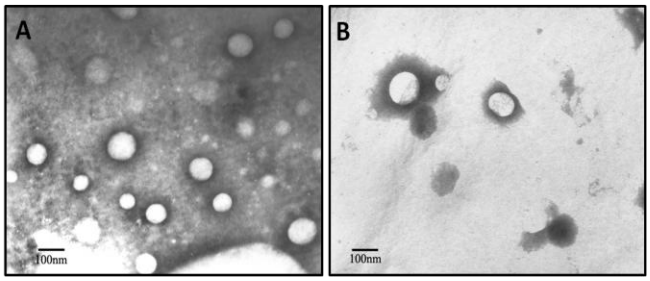

Figure 2: TEM images of PCNPs (A), PCNPs+PFG DNA(B)

3.3 *In vitro* DNA Release from PCNPs-DNA Complex

Release assay was performed in 1% PBS during 15 days incubation until entire DNA was released from the PCNPs complex. The release of DNA was at the highest level after 1-day incubation with 22% release followed by approximately 12% daily release until day8. The cumulative DNA concentration increased steadily and reached to 35% in day2, 46% in day3 and 58% in day4. The exponential release was observed between the day1 and day8, a point where the release started slowing down. The release of DNA still continued between day8 and day11, although at a low rate until it reached plateau phase after day11. Almost the entire amount of DNA was completely released by the end of day15 (see Fig. 3).

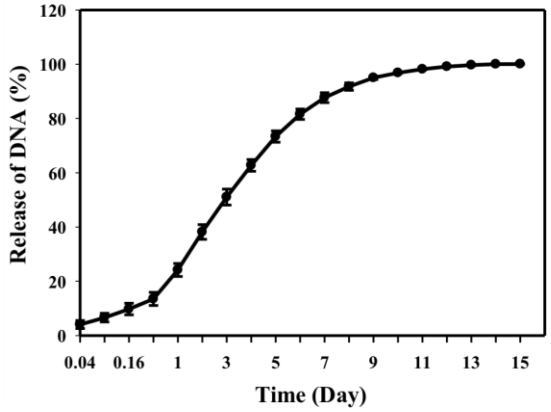

Figure 3: Release of PFG DNA from PCNPs complex

3.4 Cytotoxicity of PCNPs or PCNPs-DNA

The cytotoxicity of PCNPs and PCNPs-DNA (100µg/ml) were tested in Cos-7 cells using MTT cytotoxicity assay. Neither PCNPs nor PCNPs-DNA caused any significant toxicity to Cos-7 cells. More than 85% viability of Cos-7 cells was observed at concentrations 0.1%-1% at the end of the 3 days incubation period. We found up to 80% cell viability even at 2.5% PCNPs. However, 5% PCNPs caused 70-80% cell death. Time and concentration dependent viability was observed; shorter the incubation time (24 or 48 h) or lower the concentration more viability was seen. (see Fig. 4).

CONCLUSIONS

In conclusion, to our knowledge this is the first study suggesting successful incorporation of a DNA vaccine into pHEMA/chitosan composite nanoparticles. This complex material could be extrapolated to other areas such as drug or antibiotic delivery, biomedical research and material technologies as well.

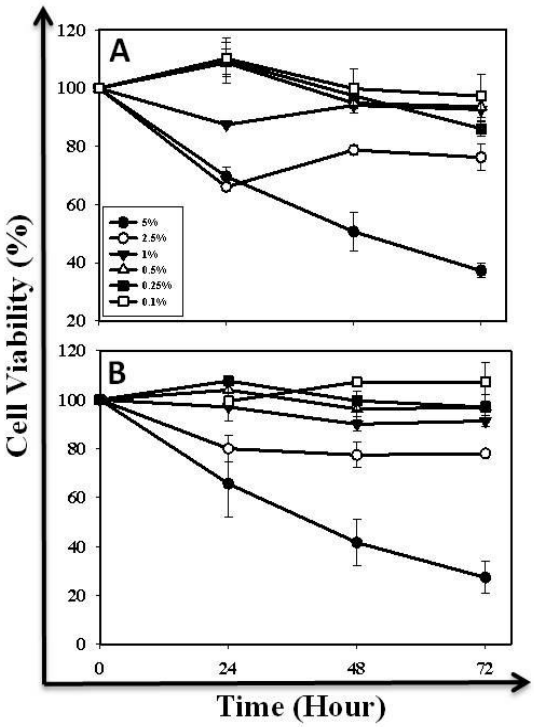

Figure 4: Viability of Cos-7 cells treated with various concentrations of PC (A) and PC encapsulated with PFG DNA (B)

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