Peptide RF482 Encapsulated Chitosan – PLGA Nanoparticles Inhibit Respiratory Syncytial Virus

S Bawage, P Tiwari, V Dennis, S Singh

Center for NanoBiotechnology Research, Alabama State University
1627, Harris way, Life science building, Montgomery
Alabama 36104, USA, ssingh@alasu.edu

ABSTRACT

Worldwide, human respiratory syncytial virus (RSV) is one of the leading causes of lower respiratory tract infections in children and older adults. Currently, an anti-viral drug ribavirin and RSV neutralizing monoclonal antibody- palivizumab are the only relief options. However, efficacy, cost and adverse effects are the problems associated with them. Nanotechnology has significantly contributed in the medicine, especially, drug delivery. Nano-encapsulation of drugs provides the advantage of longer retention and controlled release. RSV infects the host cells by F protein mediated cell membrane fusion. Chemical or protein fusion inhibitors that block this vital step of RSV infection are currently explored. In this study, we synthesized the biodegradable nanoparticles by double emulsion method, firstly we encapsulated RSV F protein derived RF482 peptide (a fusion inhibitor) with poly [lactic acid-glycolic acid] (PLGA) and then by chitosan. The size and shape of nanoparticles were determined by electron microscopy and differential light scattering. Charges were analyzed by measuring the zeta potential. Cytotoxic concentration of these nanoparticles for HEp-2 cell lines was determined by MTT assay. Fluorescence microscopy showed the attachment of nanoparticles on the HEp-2 cells. Our study showed that, the mucoadhesive chitosan-PLGA nanoparticles (without RF482 peptide) contribute in RSV inhibition. However, the RF482 peptide encapsulated chitosan-PLGA nanoparticles treatment showed higher RSV inhibition than the unloaded nanoparticles.

Keywords: RSV, Chitosan, PLGA, fusion inhibitors, nanoparticles

1 INTRODUCTION

RSV is one of the leading causes of lower respiratory tract infections in children and old adults. Currently there is no vaccine, except for the passive vaccine Palivizumab. RSV infects the cells with the help of F protein that is involved in attachment and entry of RSV into the host cell. RSV F forms a trimeric coiled-coil for fusing viral and cell membrane. This process has attracted avenues to develop fusion inhibitors (chemical and peptide) that can inhibit RSV entery into the cells [1]. There is a wide scope for the development of drugs against RSV since the drugs face the issues regarding toxicity, pharmacokinetic and pharmacodynamic stability. Various means are explored to have a reasonable mode of delivery and attain the desired results [2]. To have an efficient delivery system and protect the drugs from cellular degradation, various encapsulation methods are employed. Genereally, polymeric nanoparticles of chitosan, PLGA, polylactic acid (PLA), etc, are prefered choice for such stated task. These materials are muchoadesive, genereally biodergardable, less toxic, have controlled release properties and can act as adjuvant [3]. The present work exploits these properties and studies the effect of RF-482, a peptide fusion inhibitor encapsulated by chitosan – poly (lactic acid glycolic acid) nanoparticles (CPRF482). The nanoparticles were characterized by various biophysicsal methods and RSV inhibition was determined.

2 MATERIALS AND METHODOLOGY

2.1 Materials

Human epithelial cell lines (HEp-2) (ATCC CCL-23) were cultured in minimal essential medium supplemented with 10% fetal bovine berum (FBS), 2 mM L-Glutamine and penicillin, streptomycin and kanamycin (Sigma, USA). Respiratory Syncytial Virus (RSV) strain Long was propagated as per the instructions from ATCC. Peptide RF-482 was synthesized with the N terminal modification with H-AEEAc linker from Bacham Americas Inc.

2.2 Synthesis of Chitosan-PLGA nanoparticle

Two ml of PLGA (1%) and 500 µl (500 µg) RF-482 peptide were mixed and sonicated on ice. The resulting emulsion was added dropwise to 2% poly vinyl alcohol (PVA) and stirred for 2 h. Then 500 µl of 1% chitosan was added dropwise to stirring PVA and stired overnight and harvested at 40000 x g for 10 min. The pellet was washed twice and lyophilize. Nanoparticle stock of 1 mg/ml was prepared in MEM (2% FBS) and sonicated for 5-10 min to get uniform suspension. Control chitosan and PLGA nanoparticles were prepared similarly.

2.3 Dynamic light scattering

Nanoparticle suspension was prepared in distilled water. Hydrodynamic radius and zeta potential were measured using standard procedure for Malvern Zetasizer Nano ZS instrument.

2.4 FT-IR spectroscopy

FT-IR characteristic peaks corresponding to the nanoparticles were acquired in the spectral range of 400-4000 cm^{-1} using attenuated total reflectance (ATR) sampling accessory of Thermo Fisher Nicolet 380 instrument.

2.5 Electron microscopy

Electron microscopy was performed to image the nanoparticles. A drop of nanoparticle suspension was placed on copper grids samples and stained with 1% phosphotungstic acid. Nanoparticles were imaged using Zeiss EM10 transmission electron micrscope [4].

2.6 Cytotoxicity assay

HEp-2 cells (17000) were seeded into each well of 96 well plate and incubated at 37°C (5% CO_2) for 24 h. Cells were washed with HBSS and 100 µl nanoparticles (chitosan-PLGA and PLGA) of appropriate concentration suspended in MEM (10% FBS) were added to the cells and incubated for 48 h. MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (CellTiter 96® Non-Radioactive Cell Proliferation Assay) was performed according to the manufacturer's instruction (Promega Madison, WI). The formazon crystals were disssolved and read at 570 nm with the reference wavelength of 650 nm. The cells treated with nanoaprticles were compared with control cells (non-treated) to determine the vialbity.

2.7 Fluorescence microscopy

HEp-2 cells (30000) were seeded in an 8 chambered slide and incubated at 37°C (5% CO_2) for 24 h. Next day, the cells were washed twice with 250 µl HBSS and nanoparticles were added to the cells and incubated for 1 h. Volume was made to 250 µl/chamber with MEM (2% FBS) and incubated for 48 h at 37°C (5% CO_2). Cells were fixed with 10% TCA for 30 min and washed with 70%, 90%, 100% ethanol successively for 5 min each. Cells were then washed with 1X PBS, stained with DAPI and cell mask dyes. Cover slip was placed over the slide and sealed. The slide was observed under Nikon Ti Eclipse microscope.

2.8 RSV Inhibition

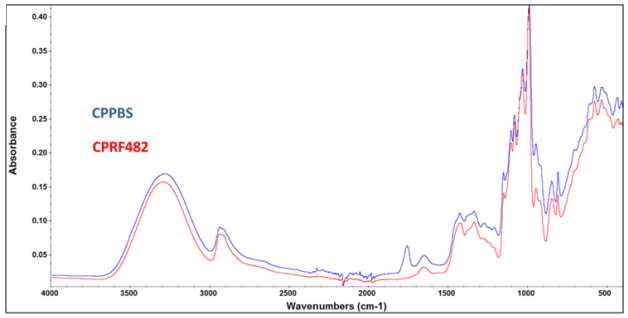
In a12-well plate, $2x10^5$ HEp-2 cells per well were seeded and incubated for 24 h. Cells were washed with HBSS before adding RSV 100 µl (100 PFU) and incubated for 1 h. Respective concentration of nanoparticles were added to the cells and total volume was made with MEM (2% FBS) to 1 ml and incubated at 37°C for 48 h. Plate was centrifuged at 3000 rpm for 5 min followed by removing the medium. Cells were trypsinized and pelleted for RNA extraction (QIAamp Viral RNA Mini Kit).

cDNA was synthesized using SuperScript® II Reverse Transcriptase (Invitrogen™) as instructed by manufacturer. Absolute quantification of RSV F gene copies was done using TaqMan chemistry, Applied Biosystem ViiA 7 real time PCR (Applied Biosystem International, Foster City, CA, USA) as previously described by Mentel et al. [5]. Each 20 µl qPCR reaction was compared with the standard curve and controls, to know the RSV F copies for the treatment groups.

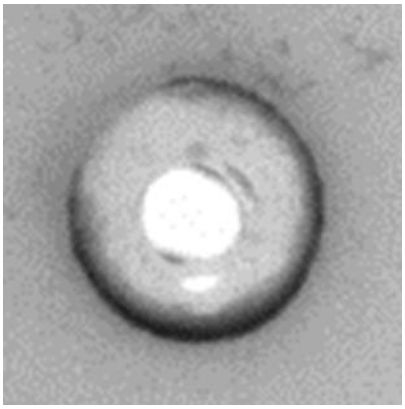
3 RESULTS

The zeta size and zeta potential was recorded and it was observed that the average size of nanoparticles encapsulating RF-482 was smaller (133.30 nm) than the control PBS encapsulating nanoparticles (212.17 nm). The zeta potential for CPPBS and CPRF482 was 37.0 ± 3.3 mV and 22.8 ± 1.3 mV, respectively. FT-IR spectroscopy showed the encapsulation of the RF-482 and TEM images show the layer of chitosan on to the PLGA, size and shape of nanoparticles (Figure 1).

A



B



C

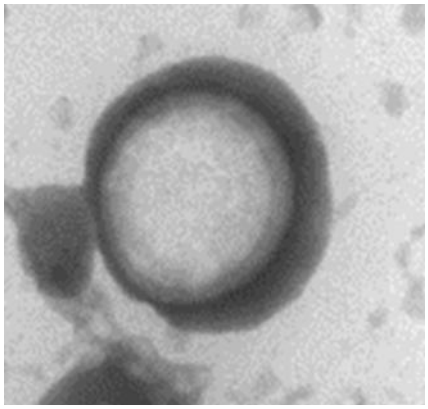


Figure 1: Synthesis and characterization of chitosan-PLGA nanoparticles to evaluate the RSV inhibition. (A) FT-IR spectrum of nanoparticles, (B) TEM image of CPPBS nanoparticle and (C) CPRF482 nanoparticle

MTT assay showed that chitosan-PLGA and PLGA nanoparticles were not toxic to HEp-2 cells up to 100 µg/ml after 48 h of exposure (Figure 2). The viability was 80% for 200 and 400 µg/ml of nanoparticles. Therefore, the nanoparticle was tested for the concentration of 25, 50 and 100 µg/ml against RSV.

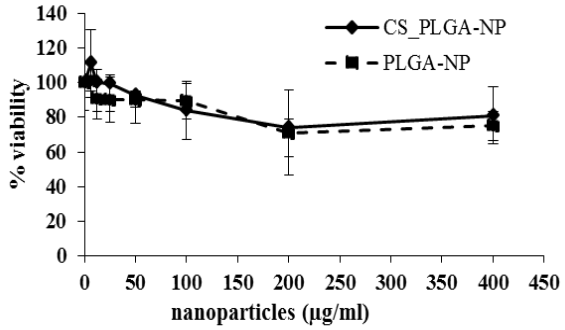


Figure 2: MTT assay for different concentration of PLGA and Chitosan-PLGA nanoparticles at 48 h

The RF-482 is 39 amino acid long and is UV invisible, hence it was FITC labeled. To know the interaction of nanoparticles with the cells fluroscence micrscopy was performed. Nanoparticles were observed adhering as aggregates over the surface of the cells (Figure 3A). Also, the uptake of CPRF482 nanoparticles makes the cell appear green (FITC labelled), as the peptide RF-482 seems distributed in the cell (Figure 3B). The nucleus appears blue due to DAPI staining and the cytoplasm appears red.

A

B

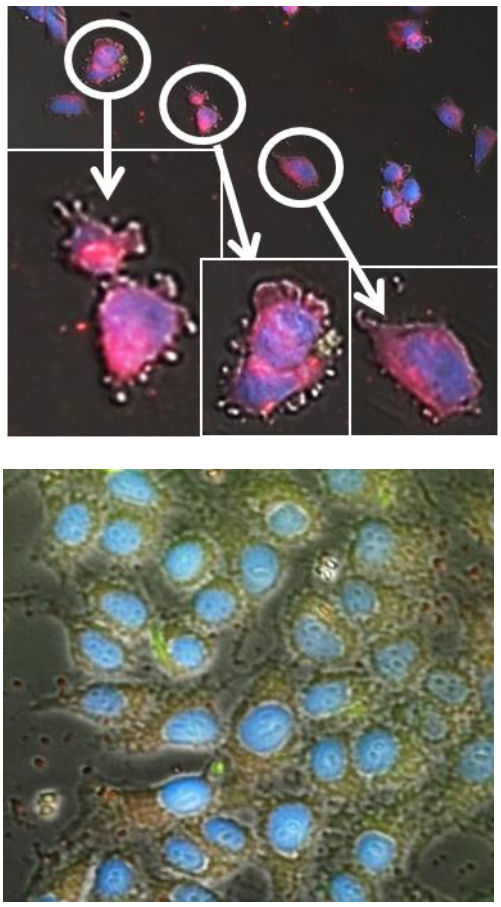


Figure 3: Uptake of chitosan-PLGA nanoparticles by HEp-2 cells after 48 h under fluorescence microscope. (A) Cells with CPPBS; inset showing nanoparticles attached to the cells; (B) cells appearing green (FTIC labeled RF-482) due to uptake of CPRF482 nanoparticles. (Blue-DAPI, Red-Cell membrane mask, Green-FTIC)

RSV was inhibited by CPRF482, i.e. chitosan-PLGA nanoparticles itself and also by RF-482 encapsulated chitosan-PLGA nanoparticles (Figure 4). The concentration of 50 and 100 µg/ml of nanoparticles (CPPBS or CPRF482) inhibited the RSV. However, at 25 µg/ml, CPRF482 nanoparticle were effective than CPPBS nanoparticles inhibiting RSV. The inhibition occurs mainly due to the fusion inhibitor RF-482, that binds the RSV F protein therby RSV can not attach to the cells. Our results also, indicates that RSV inihibition was not only due to peptide but also by the chitosan-PLGA. This inhibition can be due to the physical hinderence created by chitosan-PLGA, that does not allow RSV to interact with cell membrane. This study shows that chitosan-PLGA can be used for efficent delivery of the peptide and by itself act as an RSV inhibitor.

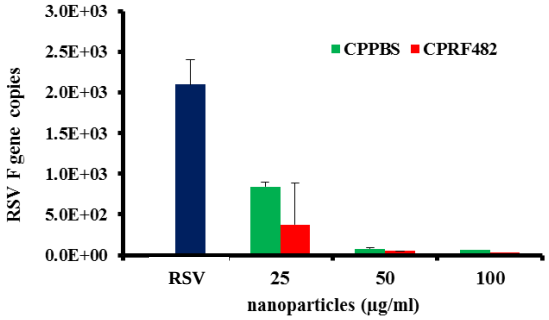


Figure 4: RSV inhibition after treatment with nanoparticles (CPPBS and CPRF482) at 25, 50 and 100 µg/ml concentration

4 CONCLUSION

Chitosan and PLGA are FDA (Food and Drug Administration) approved materials. This attracts fabrication of nanoparticles for delivery of drugs. The lower toxicity, muco-adhesive properties and controlled drug release has allured pharmaceutical research. Our study concludes that RF-482 interacts with RSV F six helix bundle and may inhibit RSV F mediated cell infection. Chitosan PLGA nanoparticles encapsulating RF-482 were characterized and found effective in inhibiting RSV at concentration of 100 µg/ml. It was surprising that chitosan-PLGA-PBS nanoparticles were also able to inhibit RSV, although the inhibition was less compared to RF-482 encapsulated nanoparticles.

REFERENCES

1. Ni L, Zhao L, Qian Y, Zhu J, Jin Z, Chen YW et al. Design and characterization of human respiratory syncytial virus entry inhibitors. Antiviral therapy. 2005;10(7):833-40.

2. Bawage SS, Tiwari PM, Pillai S, Dennis V, Singh SR. Recent advances in diagnosis, prevention, and treatment of human respiratory syncytial virus. Adv Virol. 2013;2013:595768. doi:10.1155/2013/595768.

3. Kumari A, Yadav SK, Yadav SC. Biodegradable polymeric nanoparticles based drug delivery systems. Colloids and surfaces B, Biointerfaces. 2010;75(1):1-18. doi:10.1016/j.colsurfb.2009.09.001.

4. Eroglu E, Tiwari PM, Waffo AB, Miller ME, Vig K, Dennis VA et al. A nonviral pHEMA+chitosan nanosphere-mediated high-efficiency gene delivery system. Int J Nanomedicine. 2013;8:1403-15. doi:10.2147/IJN.S43168.

5. Mentel R, Wegner U, Bruns R, Gurtler L. Real-time PCR to improve the diagnosis of respiratory syncytial virus infection. J Med Microbiol. 2003;52(Pt 10):893-6.