Silver Coated Carbon Nanotubes Inhibit RSV Infection

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ABSTRACT

Nanobiotechnology and its potential to be used in treating and diagnosing general and viral infections have allowed us to step into a new realm, when dealing with RSV. Silver with its antimicrobial effects and carbon nanotubes (CNTs) being used in certain cancer medications may provide us an insight in inhibiting RSV. Silver coated CNT nanoparticles were evaluated for their cytotoxicity on HEp-2 cells. (Human body Type-2 epithelial cells). RSV inhibition using the nanoparticles was evaluated using the Immunoflouresence and Plaque assays. Our results suggest that using 5 μg/ml or more of silver coated CNT reduced cell viability by 30%. Silver coated CNT were further studied by AFM and TEM for structural analysis. Future studies will focus on monitoring HEp-2 cells infected with RSV in presence and absence of nanoparticles using AFM.

Keywords*:* Respiratory syncytical virus, Silver coated carbon nanotubes, AFM

1. INTRODUCTION

Respiratory Syncytial Virus (RSV) belonging to the Paramyxovirus family is one of the most common causes of upper and lower respiratory tract infections. It is a leading cause of bronchiolitis and pneumonia worldwide, affecting children under the age of five, the immunocompromised, and the elderly with a high mortality rate. The RSV genome is composed of a single stranded negative sense RNA of 15,200 nucleotides encoding 11 proteins (Hacking *et al*., 2002). Among these 11 proteins only the fusion (F), which allows membrane penetration and fusion of the virus with the host cell, and the attachment (G) glycoproteins have been studied for their potential use as a vaccine. Nanoparticles such as Silver coated CNT have been applied to determine the inhibition of RSV. A concern with using nanoparticles is their cytotoxic effect. Therefore, prior to applying these nanoparticles to inhibit RSV, their cytotoxicity must be determined. The aim of this present study was to assess the cytotoxicity of the Silver coated CNT nanoparticles followed by their inhibition of RSV.

2. MATERIALS AND METHODS

2.1 Sonochemical Synthesis of Silver conjugated CNT Nanoparticle

Multi walled carbon nanotubes (MWCNT) (10 mg) were added to 60 ml dimethylformamide and magnetically stirred for 30 minutes. Then 500 mg of silver (I) acetate (Sigma Aldrich 98+ %) was added to the solution and stirred again for 10 minutes. This mixture was irradiated with a high intensity ultrasonic horn (Ti-horn, 20 kHz, 100 W/cm2) under argon gas at 13° C external cooling temperature for 72 h. The product was further washed with distilled water 3-4 times followed by drying with absolute alcohol in a vacuum overnight.

2.2 Cell and Virus

HEp-2 cells were purchased from American Type Culture collection (ATCC, Manassas, VA; CCL-23) and were propagated by standard methods using Minimum 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.

Human RSV Long strain was purchased from ATCC (VR# 26). Virulent RSV stocks were prepared and propagated in HEp-2 cells. RSV with multiplicity of infection (m.o.i) of 4:1 was added to the flask and virus adsorption was carried out for 1 h at 37^{o}C in a humidified atmosphere with 5% CO_2. MEM supplemented with 2% FBS and 2 mM L-Glutamine were added to the flask and infection of cells was observed for 72 h. RSV infected cells were harvested and cell suspensions were subjected to 2 freeze-thaw cycles at -80°C followed by centrifugation at 3,000 x g at 4°C to remove cellular debris. The viral stock was aliquoted and stored at -80°C or liquid nitrogen until further use. Viral titer of the prepared stock as

determined by plaque assay revealed a titer of 10^6 PFU / ml.

2.3 Cell cytotoxicity of nanoparticles

The cytotoxicity of Silver coated CNT nanoparticles on HEp-2 cells was determined using the Trypan Blue Assay. A stock solution of Silver coated CNT was prepared at 10 mg/ml concentration in deionized water. Concentrations of 0.25, 0.5, 1.25, 2.5, 5 and 10 µg/ml of the Silver coated CNT nanoparticle were tested. Cells were plated in triplicates in 12 well plates. After 24 h different concentrations of nanoparticles were added to the cells and incubated for 24, 48, 72 h respectively. After each time frame the cells were washed with HBSS and trypsinzed. The cells were centrifuged and washed twice before 100 µl were mixed with the Trypan Blue stain. This mixture of the cells and Trypan Blue was then placed into a hemocytometer and viable or non viable cells were counted by a light microscope. Non viable cells stained blue while viable cells did not take the stain. The viability of both cell lines was determined as the percentage of live cells out of the total number of cells counted.

2.4 Immunofluoresence

Once the cytotoxicity of the Silver coated CNT nanoparticles was determined in both HEp-2 and Vero cell lines, we were able to concentrate on determining the inhibition of RSV using the nanoparticles. The nanoparticle samples at concentrations of 0.25, 0.50, 1.25, 2.5, 5, and 10 µg/ml were mixed with 100 PFU of RSV and added to the cells. After plating the cells in an 8-chamber slide and incubation for 24 h, RSV and desired concentrations of Silver coated CNT nanoparticles were added to each chamber and incubated for 48 h. This was followed by fixing the cells with 10%TCA for 15 minutes and washed with 70%, 90%, and absolute alcohol for 5 minutes each. The slides were then washed three times in 1X PBS for 5 minutes each. Washed cells were blocked using 0.5ml of 3% dry milk powder in PBS for 30 minutes followed by washing with 1X PBS three times for five minutes each. Polyclonal goat anti-RSV antibody (Chemicon) in antibody buffer (2% dry milk in PBS) were added to the cells, and incubated for 1 h at room temperature. The cells were washed three times again with 1X PBS followed by the addition of the secondary antibody, rabbit anti-goat Ig (H+L) (Southern Biotechnology) diluted 1:2000 in 2% dry milk powder in PBS. After addition of secondary antibodies the cells were incubated for another h in the dark. Following the incubation, 75µl of DAPI stain was added to the cells and incubated for 5 minutes. Cells were visualized with a Nikon fluorescent microscope (Model Ti-U Phase, Lewisville, TX).

2.5 Plaque Assay

HEp- 2 cells were plated ($2.2x10^5$ cells in MEM-10) in a six well plate and incubated for 48 h to get 80-90% confluency. The nanoparticles in various concentrations were prepared in 100 µl of MEM-0. RSV (100 PFU) was added to each concentration of nanoparticles, and incubated for 30 minutes at room temperature. The media was aspirated from the 6 well plate and cells were washed with 1X PBS. The RSV and nanoparticle mixtures (100 µl) were added to respective wells and the plates were rocked gently. Plates were incubated for 1 h at 37°C under 5% CO2 in a humid atmosphere, Cells were then overlayed with 0.75% methylcellulose (2 ml/well) in DMEM containing 2% FBS. The cells were incubated for 4-5 days. After incubation the methylcellulose was aspirated from the wells and 1.0 ml of cold methanol was added and incubated at -20°C for 1 h. The methanol was removed and the cells were stained using 1ml Crystal Violet Stain. Excess stain was removed and the plates were dried by inversion. Plaques were counted in the purple monolayer using a dissecting microscope.

2.6 Transmission Electron Microscopy (TEM) analysis

High resolution TEM (HRTEM) was performed using a JOEL-2010 machine. Pictures were taken at 50,000-125,000 magnifications.

2.7 Atomic Force Microscopy (AFM) analysis

AFM pictures were obtained using the NANOSCOPE-R2 AFM (Pacific Nanotechnology, Santa Clara, CA, USA). Silver coated CNT were mixed with 100 PFU of RSV and allowed to incubate for 30 minutes before being inactivated using formalin. Ten microliters of the mixture was placed onto a slide, dried and visualized under the microscope. Close contact mode and standard silicon cantilevers (Pacific Nanotechnology, Santa Clara, CA, USA) 450 µm in length and 20 µm in width were employed for imaging. The cantilever oscillation frequency was tuned to the resonance frequency of approximately 256 kHz. The set point voltage was adjusted for optimum image quality. Both height and phase information were recorded at a scan rate of 0.5 Hz, and in 512 x 512 pixel format.

3. RESULTS

3.1 TEM and AFM analysis

Nanoparticles were characterized microscopically using TEM and AFM. TEM was performed in order to analyze the size and shape of nanoparticles. TEM micrographs revealed that the Silver coated CNT nanoparticles had a size of ~10 nm with nanotubes ~ 30 nm (Figure 1a). TEM was confirmed with AFM images (Figure 1b).

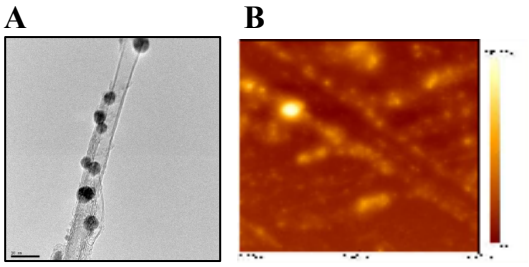

Figure 1: (A) Surface visualization of Silver coated CNT nanoparticles using Transmission Electron Microscopy (TEM) (B) Atomic Force microscopy (AFM) 2D image of Silver coated CNT nanoparticles

3.2. Cytotoxicity analysis

The percent viability of both cell lines was determined using the Trypan Blue Assay. The results show that at 1.25 µg/ml the cell viability was 85 % which decreased to about 70 % at 5µg/ml (Figure 2).

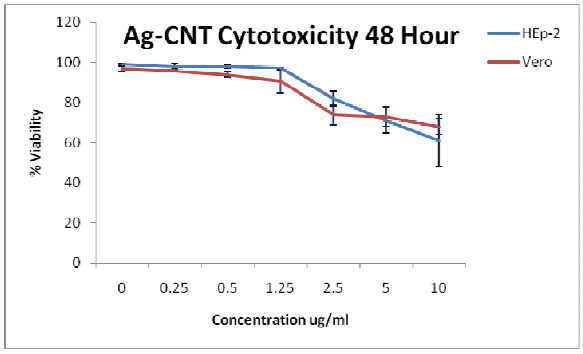

Figure 2: Trypan Blue cytotoxicity of Silver coated CNT with HEp-2 cells (blue) and Vero cells (red).

3.3. Immuno-Fluorescence Studies

Nanoparticles were mixed with 100 PFU of RSV and added to the cells to determine the inhibition of infection. Reduction in cytopathic effects (syncytia) in HEp-2 cells was observed to determine inhibition of RSV infection by Silver coated CNT. The cells infected with RSV showed marked syncytia formation (data not shown) and extensive immunofluroscence (Figure 3a). Cells infected with RSV mixed with Silver coated CNT nanoparticles clearly show significant reduction in RSV infection compared to the cells infected with RSV alone (Figure 3b).

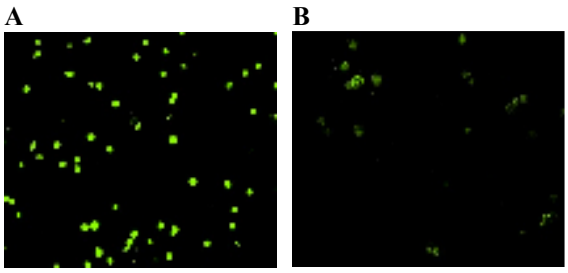

Figure 3: RSV inhibition by immunofluroscence (a) Cells infected with RSV (b) Cells infected with RSV mixed with Silver coated CNT nanoparticles (1.25µg/ml)

The results of the present study show that Silver coated CNT nanoparticles can inhibit RSV infection. This approach may prove valuable in developing a therapeutic regime for RSV, and perhaps other dangerous infections including HIV.

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