CNTs and Silver coated CNTs provide immune responses against RSV

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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 Respiratory synctical virus (RSV). RSV has long been considered a major respiratory tract pathogen of children causing lower respiratory tract infections and pneumonia. In the present study**,** Silver coated CNTs and CNTs were evaluated for their cytotoxicity on HEp-2, and Vero using MTT assays. The cells were incubated with various concentrations of the nanoparticles and assessed for the cell viability at 24, 48, and 72 hours. The results show that at 1.25 µg/ml the cell viability is at 85 % which decreased to about 65 % at 5µg/ml after 48 h post-transfection. HEp-2 and Vero cells were infected with RSV in presence and absence of nanoparticles and inhibition effect of the nanoparticles on RSV was evaluated using immunofluorescence. Additionally, the characteristics of the nanoparticles were evaluated using AFM, TEM. Moreover, the immunological response of the nanoparticles is currently being analyzed using HEp-2 cell line.

Keywords*:* Respiratory syncytical virus, Carbon Nanotubes (CNTs), Silver coated CNTs, 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 [1]. 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 CNTs and Silver coated CNTs 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 CNTs and Silver coated CNTs followed by their inhibition of RSV. Subsequently, we studied the nanoparticles effect on the gene expressions.

2. MATERIALS AND METHODS

2.1 Sonochemical Synthesis of Silver coated CNTs

Multi walled carbon nanotubes (CNTs) were purchased from Nanostructured & Amorphous Materials. These CNTs are < 95% pure (stock #1205YJ) with a density of 0.04-0.05 g/cm3. CNTs (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.

2.3 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.

2.4 Atomic Force Microscopy (AFM) analysis

AFM pictures were obtained using the NANOSCOPE-R2 AFM (Pacific Nanotechnology, Santa Clara, CA, USA). CNTs and Silver coated CNTs were 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.

2.2 Cell cytotoxicity of nanoparticles

The cytotoxicity of CNTs and Silver coated CNTs on HEp-2 cells was determined using the MTT dye reduction assay in Vero and HEp-2 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 (0.25, 0.5, 1.25, 2.5, 5 and 10 µg/ml) of CNTs and Silver coated CNTs. After 24, 48 and 72 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

2.3 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°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.

2.4 Immunofluoresence

Once the cytotoxicity of the CNTs and Silver coated CNTs were 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, and 5µg/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 CNTs and Silver coated CNTs 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. Washed cells were blocked using 0.5ml of 3% dry milk powder in PBS for 30 minutes. 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). Following secondary antibody incubation, the cells were stained with DAPI.Cells were visualized with a Nikon fluorescent microscope (Model Ti-U Phase, Lewisville, TX).

2.5 Gene Expression of HEp-2 cells Treated with Nanoparticles

HEp- 2 cells were plated ($1x10^6$ cells in MEM-10) in a six well plate and incubated for 24 h to get 80-90% confluency. The nanoparticles at 2.5µg/ml concentrations were mixed with RSV (100 PFU) and incubated for 30 minutes at room temperature. The RSV, nanoparicles alone and nanoparticle+RSV mixtures (100 µl) were added to respective wells and the plates were rocked gently. The cells were incubated for 2 hours and then the media was aspirated from the 12 well plates and added more MEM -10 media and incubated for 16hr,24hr and 48hr at 37°C under 5% CO2 in a humid atmosphere.

2.6. Measurement of cytokine concentrations

Cytokine ELISAs were performed as previously described [2]. Concentrations of IL-8 and IL-6 cytokines were quantified in cell cultures supernatants using Opti-EIA sets (BD-PharMingen, San Diego, CA) according to the manufacturer's instructions.

3. RESULTS

3.1 TEM and AFM analysis

Nanoparticles were characterized in order to analyze the size and shape microscopically using TEM and AFM. TEM and AFM analysis revealed that the CNTs 10-20 nm in diameter and ~15 µm in length and Silver coated CNTs had a size of ~10 nm with nanotubes ~ 30 nm (Figure 1).

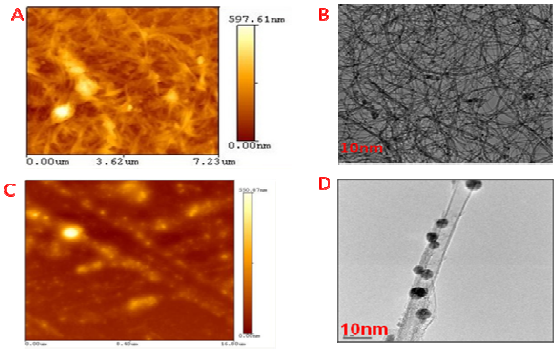

Figure 1: a) AFM 2D image of CNTs b) TEM images of CNTs c) AFM images of Silver coated CNTs d) TEM image of Silver coated CNTs. CNTs- 10-20nm in diameter and 0.5-20mm in length. Silver nanoparticles attached to CNTs are 5-10nm in diameter.

3.2. Cytotoxicity analysis

The percent viability of both cell lines was determined using the MTT Assay. The results show that at 1.25 µg/ml CNTs, the HEp-2 cell viability was 85 % which decreased to about 70 % at 5µg/ml after 48 and 72h post infection (Figure 2a). For the Vero cell lines, cell viability was 85% even at 5µg/ml after 48 and 72h post infection.(Figure 2b).Moreover, our results showed that Silver coated CNTs were more toxic then CNTs at the concentration of 5 µg/ml (cell viability was about 60%) after 72 h post-infection in HEp-2 cell lines. For the Vero cell lines, cell viability decreased to about 50% at 5µg/ml after 72h post infection. .

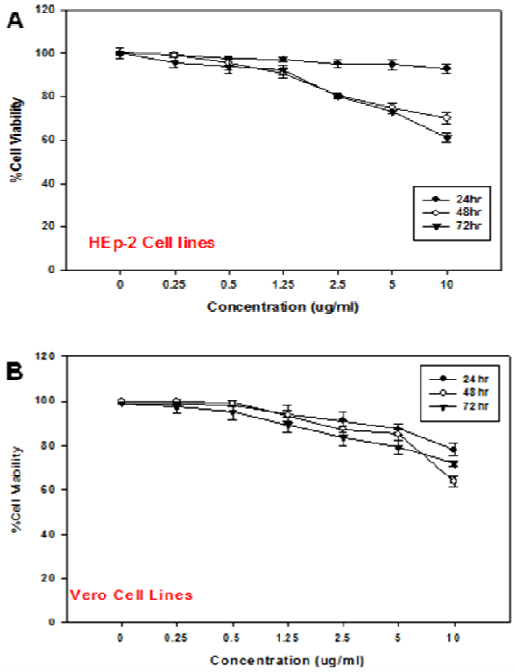

Figure 2: MTT cytotoxicity assay of CNTs with HEp-2 cells (a) and Vero cells (b).

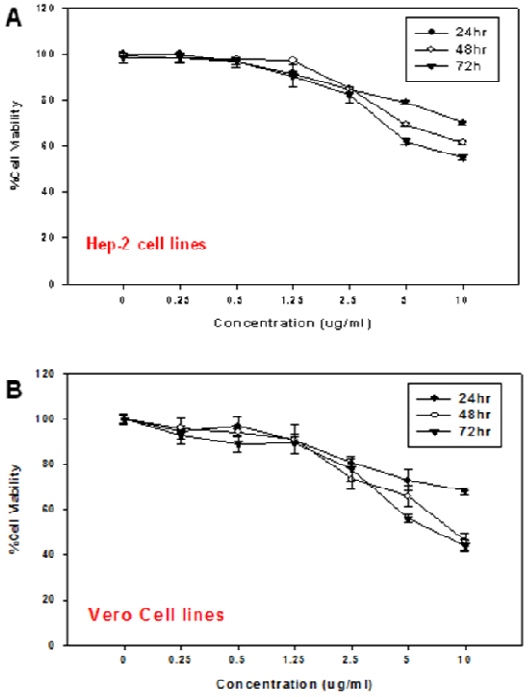

Figure 3: MTT cytotoxicity assay of Silver coated CNTs with HEp-2 cells (a) and Vero cells (b).

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 CNTs and Silver coated CNTs. The cells infected with RSV showed marked syncytia formation (data not shown) and extensive immunofluroscence (Figure 4). Cells infected with RSV mixed with CNTs and Silver coated CNTs nanoparticles clearly show significant reduction in RSV infection compared to the cells infected with RSV alone (Figure 4a-b).

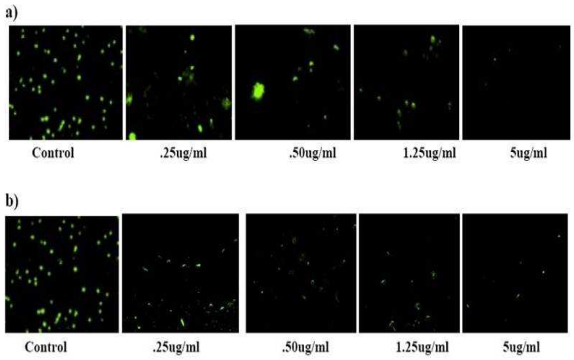

Figure 4: RSV inhibition by immunofluroscence. **Control:** Cells infected with RSV a) Cells infected with RSV mixed with Silver coated CNTs (b) cells infected with RSV mixed with CNTs

3.4. Gene Expression of HEp-2 cells Treated with Nanoparticles

Figure 5 shows the gene expression of HEp-2 cells treated with Nanoparticles, RSV and RSV mixed with Nanoparticles for 24 h and 48h.It was evident that IL-6 and IL-8 up-regulated after treatment with RSV+ Silver coated CNTs. For the same period, the expression of these genes in the control of HEp-2 cells and RSV groups were relatively lower. Our results also showed that when we increased the incubation time from 24h to 48h, the gene expression was also increased. Moreover, the Silver coated CNTs also up-regulated IL-8 expression relatively higher compared to HEp-2 cells and CNT groups (Figure 5). In conclusion, we can say that the Silver coated CNTs might activate some pathways or inhibit RSV affect so it increased some of the gene up-regulations.

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

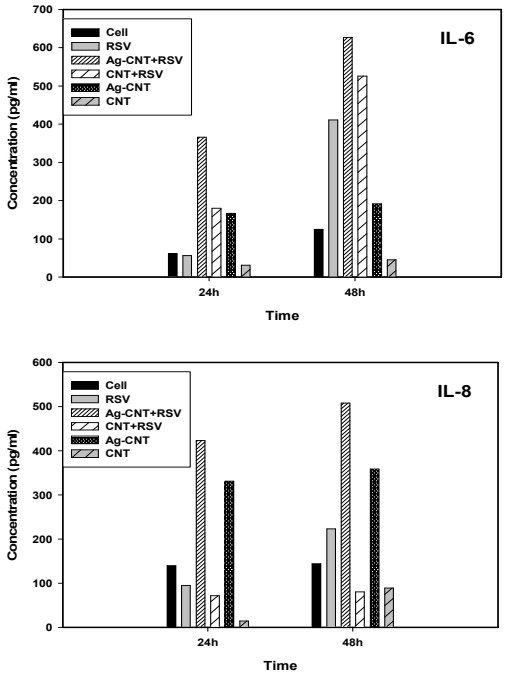

Figure 5: Gene expression of IL-6 and IL-8 in HEp-2 cells incubated with culture medium containing nanoparticles at 2.5µg/ml, RSV and RSV mixed with nanoparticles for 24 h and 48h.

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