Respiratory Syncytial Virus Inhibition by Gold and Titanium Nanoparticles

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ABSTRACT

Nanoparticles have been gaining extensive usage in medicine and therapy. The structural characteristics of nanoparticles and their interactions with surface modifiers are essential to their functions. We used gold and titanium nanoparticles to study the inhibition of RSV in cell culture. Respiratory Syncytial Virus (RSV) is the leading cause of severe respiratory illnesses including bronchiolitis and pneumonia in young children. The characteristics of nanoparticles were evaluated by Transmission electron microscopy (TEM) and Atomic Force Microcopy (AFM). TEM and AFM micrographs of gold and titanium nanoparticles revealed that they had a size of 15-50nm. The nanoparticles were analyzed for cell toxicity, and were found to have low toxicity in HEp-2 cells. The nanoparticles were then evaluated for their effectiveness to inhibit RSV. Our results revealed that gold and titanium nanoparticles inhibited RSV infection as estimated by immunofluorescence assay and by viral titer assays. Studies are underway to determine interactions of nanoparticles with RSV surface proteins interactions.

Keywords: Respiratory Syncytial Virus, nanoparticles, gold, titanium

1 INTRODUCTION

Respiratory Syncytial Virus is the leading cause of severe respiratory illnesses such as bronchiolitis and pneumonia in young children. RSV is a Paramyxovirus with negative-sense genomic RNA that encodes for eleven proteins, two of which, F and G are major surface proteins. The G protein is responsible for viral attachment to the host cell, while the F protein facilitates viral entry and spread of the virus from infected to normal cells leading to syncytia formation. Nanoparticles have been gaining extensive usage in medicine and therapy. The structural characteristics of metal nanoparticles and their interactions with surface modifiers are essential to their functions[1], as they should be stable enough to work with at ambient conditions. Silver nanoparticles in particular have strong antimicrobial functions which show potential as antimicrobial agents[2]. Silver nanoparticles have also been shown to attach to the gp120 surface glycoprotein of HIV and inhibit infection3. As the gp120 surface protein of HIV is similar to the G protein of RSV in structure and function, we hypothesized that the nanoparticles would attach to the G protein on the surface of the virus and block the sites responsible for viral attachment to the cell and thus inhibiting RSV infection4. In the present study, we used gold and titanium nanoparticles to study the inhibition of RSV infection in human cell lines.

2 MATERIALS & METHODS

2.1 Nanoparticles

Gold nanoparticle colloidal solution was bought commercially from Nanoparts™ Inc. (Hawaii, USA) with a concentration of 1.51 x 10e + 11 particles / ml.

Titanium nanoparticles were synthesized by Dr Ashok Kumar's group From Department of Mechanical Engineering, University of South Florida, Tampa, Florida, USA. Titanium stock solution was prepared after sonication.

2.2 Characterization of the Nanoparticles

The synthesized nanoparticles were analyzed using TEM (JEOL-2010 microscope). A small drop (20-30 µL) of each sample was placed on top of a formvar/carbon coated copper grid. The drop of sample was allowed to air dry. Once dry, the grid containing the sample was placed into TEM and viewed. Pictures were taken at 50,000-125,000 magnifications. Samples were confirmed by Energy dispersive X-ray (EDS) analysis. The XRD measurements were carried out with a Rigaku-D/MAX-2200.

Three-dimensional topography and physical properties of a surface can be imaged by Atomic Force microscopy (AFM) analysis. Approximately 10 µl of nanoparticles solution (50 µg) were deposited

onto a freshly cleaved piece of mica and air-dried for 30 min under a clean, dry airflow until the surface appeared dry. The dried mica with samples was used for AFM (Nanoscope R2, Pacific Nanotechnology, Santa Clara, CA, USA) studies. 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. AFM images containing nanoparticles in a large scanning area were processed using NanoRule software (Pacific Nanotechnology, Santa Clara, CA, USA).

2.3 Cells 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 was added to the flask and infection of cells was observed for 3 days. RSV infected cells were harvested and cell suspension was subjected to 2 freeze-thaw cycles at -80^{o}C followed by centrifugation at 3,000 x g at 4^{o}C to remove cellular debris. The viral stock was aliquoted and stored at -80^{o}C or under liquid nitrogen until further use. Viral titer of the prepared stock was determined by plaque assay and showed a titer of 10^5 PFU / ml.

2.4 Cell Cytotoxicity of Nanoparticles

The effect of nanoparticles on cytotoxicity was measured by MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) dye reduction. HEp-2 cells were seeded in a 96-well plate at a density of 20,000 cells per well in MEM containing 5% FBS and grown overnight. After 24 h nanoparticles at different concentrations were added to the cells. After 48 h post-incubation with nanoparticle complexes, media were removed and cells were washed twice with sterile PBS. 10 µl of MTT (5 mg/ml) in sterile filtered PBS was added to each well and then incubated for 4 h to allow formation of formazan crystals at 37°C. After 4 h, 150 µl of DMSO was added to each well to dissolve the MTT formazan crystals and the plate was incubated at 37°C for 30 min. The absorbance of formazan products was measured at 490 nm using a microplate reader.

2.5 RSV Inhibition Studies

Nanoparticle samples at various concentrations were tested for RSV inhibition. The nanoparticles at different concentrations were mixed with 100 PFU of RSV and incubated for 30 minutes at room temperature. The nanoparticle + RSV mixture was then added to 60-70% confluent HEp-2 cells. HEp-2 cells infected with RSV without nanoparticles were used as a positive control.

The inhibition of RSV infection by each nanoparticle sample was analyzed visually using an inverted light microscope and by indirect immunofluorescence. RSV inhibition was also determined by estimating viral titers by performing plaque assay.

For indirect immunofluorescence HEp-2 cells were grown in 8 chambered slides (BD Biosciences) for 24 h to 60% confluence. The cells were washed with phosphate buffer saline (PBS) and fixed using 10% trichloroacetic acid for 15 min. The 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 washes with PBS. The fixed cells were incubated for 1 h at room temperature with Polyclonal goat anti-RSV antibody (Chemicon) in antibody buffer (2% dry milk in PBS). The cells were washed with PBS and then incubated for 1 h at room temperature with FITC-conjugated rabbit anti-goat Ig (H+L) secondary antibody (Southern Biotechnology) in antibody buffer. Cells were visualized with a Nikon fluorescent microscope (Model Ti-U Phase, Lewisville, TX).

RSV inhibition was also determined by viral titer estimations. HEp-2 cells (1.2 x 10^5 cells/well) were plated in 12-well plates (Corning) in 1 ml MEM with 10% FBS. After 24 h, prepared nanoparticle samples + RSV mixtures were added to the cells and allowed to absorb for 1 h at 37^{o}C in CO_2 incubator. Cells were then overlayed with 0.75% methylcellulose in DMEM containing 2% FBS. Cells were observed for plaque formation 5 days after incubation. The cells

were fixed with methanol for 1 h at -20°C and then stained with 0.1% crystal violet. The plaques were counted using a microscope at 40X magnification.

3 RESULTS AND DISCUSSION

3.1 Characterization of Nanoparticles

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 gold nanoparticles had a size of ~20-30nm (Fig. 1) EDS studies confirmed that all the particles were either gold or titanium and there were no impurities found in the sample. AFM images of the gold nanoparticles confirmed that the particles ranged from 15 to 30nm (Fig. 2a). AFM micrographs of titanium particles revealed that they had a size of ~50nm (Fig. 2b). Titanium particles showed agglomeration.

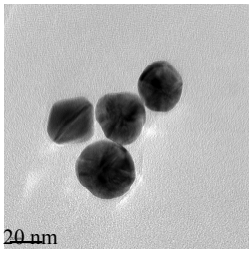

Figure 1: TEM micrograph of gold nanoparticles

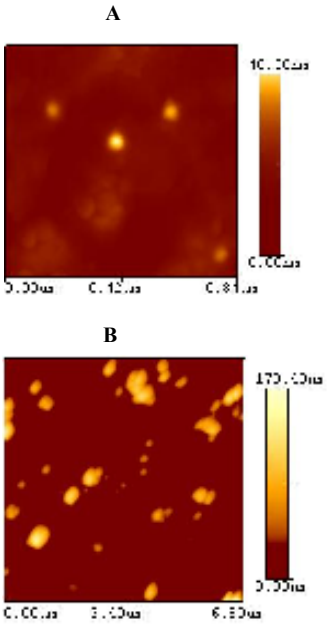

Figure 2: AFM micrograph of (a) gold nanoparticles (b) titanium nanoparticles

3.2. Microscopic analyses of nanoparticles and RSV mixture

We analyzed the prepared nanoparticles and RSV mixture to establish the attachment region of nanoparticles on the virus. AFM studies indicated that RSV particles were almost round in shape. Even though the dimensions of the RSV particle exhibited a polymorphous distribution via off-line particle analysis of AFM, most of the RSV particles had a diameter of approximate 135nm (Fig. 3a). AFM pictures revealed gold particles around the virus surface (Fig. 3b). AFM pictures of nanoparticles attached to RSV show different sizes compared to nanoparticles alone. AFM pictures of nanoparticles attached RSV show that the virus is much smaller after nanoparticle attachment

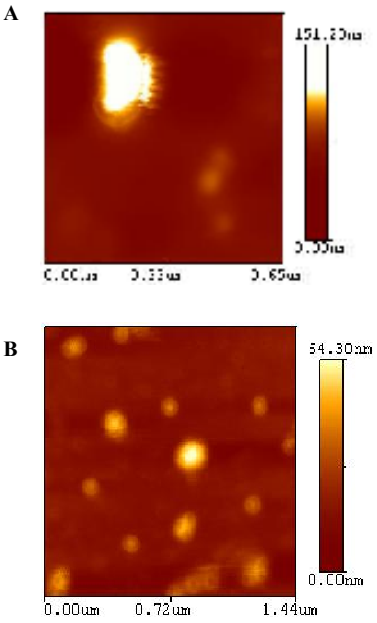

Figure 3: Atomic Force microscopy (AFM) 2D image of (a) RSV (b) RSV with gold nanoparticles after 30 min. incubation

3.3 Cytotoxicity Analysis of Nanoparticles

Gold and titanium nanoparticles were tested at different concentrations for toxicity to HEp-2 cells by the MTT assay. Gold nanoparticles showed 16% reduction in cell viability at 2.5 µl and 26.4% at 25 µl concentration (Fig. 4a). Titanium nanoparticles showed 17% reduction in cell viability at 50 µg/ml (Fig. 4b).

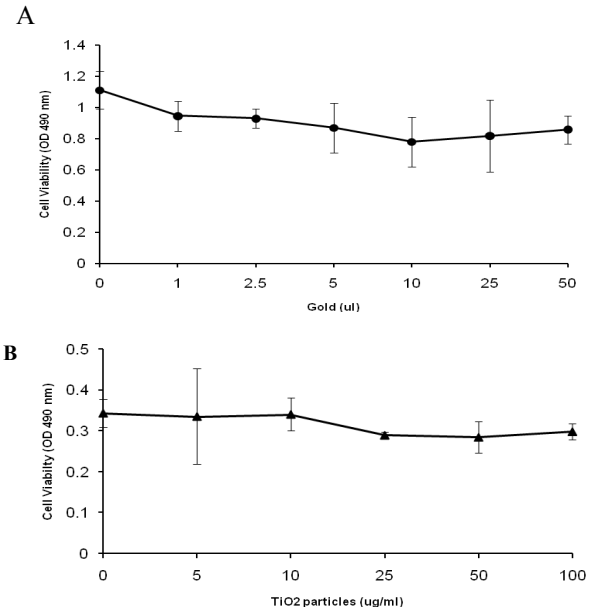

Figure 4: MTT assay for 48 hr cytotoxicity of nanoparticles to HEp-2 cells (a) gold nanoparticles (b) titanium nanoparticles

3.4 Inhibition of RSV Infection

Reduction in cytopathic effect (syncytia formation) in HEp-2 cells was used to determine the extent of RSV infection. The cells infected with RSV alone showed marked syncytia formation. Inhibition of RSV infection was also confirmed by performing indirect immunofluorescence on RSV mixed with nanoparticles infected HEp-2 cells. Cells infected with RSV mixed with gold and titanium nanoparticles clearly showed significant reduction in RSV infection (seen as reduction in immunofluorescence) as compared to the cells infected with RSV alone (Fig. 5).

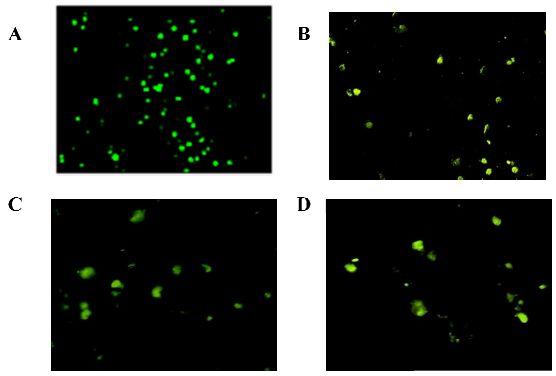

Figure 5: RSV inhibition by immunofluorescence. Cells infected with RSV (a) no nanoparticles (b) mixed with gold nanoparticles (25 µl) (c) mixed with 25 µg/ml titanium nanoparticles (d) mixed with 50 µg/ml titanium nanoparticles

RSV inhibition was also determined by performing the plaque assay on HEp-2 cells. Viral titers were reduced by 67% at 50 µg/ml titanium nanoparticles (Fig. 6).

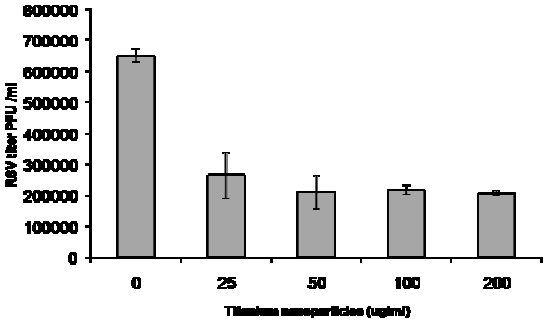

Figure 6: RSV inhibition by titanium nanoparticles as estimated by the plaque assay.

The results of the present study show that nanoparticles can inhibit RSV infection can be used in future for therapeutic purposes.

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