miRNA Expression Analysis in HEp-2 Cells after Gold Nanorod Application

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ABSTRACT

Gold Nanoparticles are popular because of their use in drug delivery and other biomedical applications. The purpose of the present study was to evaluate the toxicity of GNRs to HEp-2 cells. In the present study, 20nm GNRs were used and their toxicity to the cells was tested using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and Trypan Blue Cell Exclusion assay. MTT results showed 90% cell viability at 10 µg/mL compared to 56% at 50 µg/mL in 72 hours which was similar to Trypan Blue Assay. We synthesized fluorescein-conjugated GNRs and investigated its localization in the cells. With the use of immunofluorescence microscopy, we observed that the GNRs entered the cells at 4 hours. We furthermore investigated the effect of GNRs on gene expression by microRNA (miRNA) expression analysis of cells. miRNA PCR array revealed upregulation of majority of genes in response to GNRs after 4 hr. incubation. The purpose of this study was to assess how the GNRs affected the cells at the molecular level to facilitate prospective applications.

Keywords: Gold nanoparticles, miRNA, Cell viability, gene expression

1. INTRODUCTION

Nanotechnology has been a cornerstone for recent cutting-edge biological research and other areas of science. Nanoparticles (NPs) have made it possible to incorporate effective approaches on molecular levels such as drug delivery, bioimaging, and other biological applications.[1] Gold nanorods (GNRs) are have been used for drug delivery and other cellular uses. With low cytotoxicity and functionalization abilities, GNRs are ideal for biological uses and have opened up avenues in nanotechnology for various applications. [2-4]

miRNAs are non-coding RNAs of 22 nucleotides in length and function in cellular translation and degradation. miRNAs are transcribed by RNA Polymerase II and have been found to be included in the expression of various types of human cancers.[5] Through base pairing with mRNAs, miRNAs are able to complete its task of either cleaving the mRNA, or inhibiting translation. The function of miRNAs makes them ideal for further evaluation in effective translocation of NPs within cells, and possible cell marking and gene manipulation.[6]

The current study evaluates the effect of GNRs on human cells (HEp-2) to investigate its localization into the cell, cytotoxicity and effect on gene expression to further research and biological studies.

2. MATERIALS & METHODOLOGY

2.1 Materials

HEp-2 cells were purchased from ATCC® and cultured with L-Glutamine, 10% fetal bovine serum, and Antibiotic Antimycotic Solution which were purchased from Sigma-Aldrich®. GNRs were purchased from Nanopartz, Inc. ©. miRNeasy Mini Kit, miScript II Reverse Transcription Kit, and miScript PCR Arrays were purchased from Qiagen© for gene expression studies.

2.2 Cell Culture

Human epithelial (HEp-2) cells were cultured using minimal essential medium (MEM) with 10% fetal bovine serum (FBS) in T-75 flasks and placed in incubator with temperature of 37°C with 5% CO_2.

2.3 Characterization of GNRs

GNRs procured from Nanopartz were 20 nm in size and functionalized with carboxyl group. GNRs were characterized using Malvern Zetasizer Nano ZS. Fourier transform infrared spectroscopy (FT-IR) was conducted using Thermo Fisher Nicolet 380.

2.4 Cell Viability with Nanoparticles

The cytotoxicity of nanoparticles was determined by MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) dye reduction assay using CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) kit (Promega).

The cytotoxicity was also determined using Trypan Blue Cell Exclusion Assay. Cells ($9.5x10^4$/ well) were seeded in 48-well plates and incubated for 24 hours. GNRs at different concentrations were added and incubated for 72 hours. Cells were trypsinized, resuspended in PBS and counted after mixing with

trypan blue dye. Cells were counted on AMG All-In-One Digital Inverted Fluorescence Microscope using hemocytometer.

2.5. GNR Functionalization

GNRs were functionalized using EDC-NHS chemistry. 500µL of EDC (1mg/mL) and 500µL of NHS-Fluorescein (2mg/mL) were added to 100uL of GNR (325µg/mL) and vortexed on shaker for 30 minutes. The solution was centrifuged at 12000 rpm for 30 minutes. The pellet was washed with 1ml distilled water twice and centrifuged. GNR were resuspended in 1ml RNAse free distilled water and stored at 4^{o}C till use.

2.6. Cellular uptake of GNRs

Cells (3 x 10^4/well) were seeded in 8 chamber slide and incubated for 24 hours. After 24 hours, GNRs (20µg/mL) was added to each well and incubated for 2, 4, 6, and 24 hour time periods. After each time interval, cells were fixed with 10% TCA solution for 30 minutes and washed with 70%, 90%, and 100% ethanol for 5 minutes respectively. Cells were stained with DAPI stain and GNR uptake and localization was observed using Nikon Ti Eclipse immunofluorescent microscope at a total magnification of 200X magnification.

2.7. Gene Expression Studies

Cells (2x10^5/ well) were seeded in 12 well plate and incubated at 37°C for 24 hours. GNRs (5µg/mL) were suspended in MEM-10 and incubated with cells for 4 and 24 hour time periods. Cells were trypsinized and pelleted. RNA was extracted using miRNeasy kit and cDNA was synthesized with miScript II reagents according to the manufacturer`s protocol (Qiagen). miScript miRNA PCR Arrays were performed for gene expression analysis using Applied Biosystems® ViiA™ 7 Real Time PCR Systems. Data was analyzed using Qiagen PCR Array Data Analysis Tool.

3. RESULTS & DISCUSSION

3.1. Characterization of GNRs and GNR-FITC

Transmission Electron Microscopy (TEM) showed GNRs being 20nm in length. Zeta potential analysis showed surface charge value -20.8 mV in GNRs and -34.7 mV in GNRs-Fluorescein confirming conjugation of the fluorescent marker on the NPs (Fig. 1).

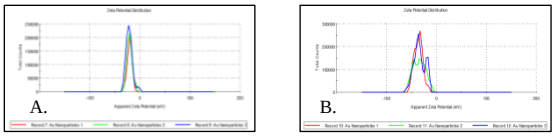

Fig. 1. A). Zeta Potential Analysis for GNRs. Average ZP value= -20.7667 mV. B). Zeta Potential Analysis for FITC-Conjugated GNRs. Average ZP=-34.7333 mV.

The FT-IR spectroscopy was conducted for GNRs alone and GNRs-Fluorescein (Fig. 2). FT-IR analysis further confirmed the conjugation of GNRs and Fluorescein.

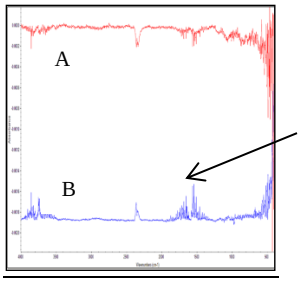

Fig. 2. FTIR analysis of (A.) GNRs (Red) (B.) FITC-GNRs (Blue).

3.5 Cell Viability with GNRs

Toxicity of GNRs to HEp-2 cells was investigated microscopically (Fig. 3). We also used standard MTT assay to check the cytotoxicity of GNRs. Concentrations ranging from 0 to 50 µg/mL of nanoparticle sample were tested for toxicity to HEp-2 cells.

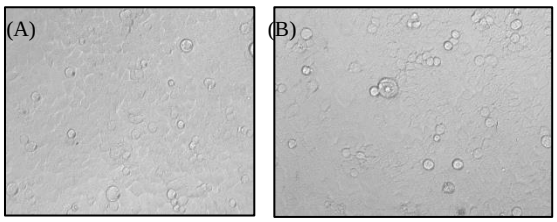

Fig.3. Morphological analysis (A.) Control (B.) GNR 50 µg/ml post 72 hr incubation

In vitro incubation of Hep-2 cells with and without GNR 50 µg/ml showed remarkable morphological alterations (Fig.3.) and cell death post 72 hrs. characterized by disruption of monolayer. It caused a dose-dependent cytolytic and nuclear change in cell morphology. Although, MTT Assay results after 24 hour incubation with GNRs showed approximately 80% cell viability at 10µg/mL, concentrations of 50µg/mL showed a cell viability of 55% after 72 hour incubation (Fig.4).

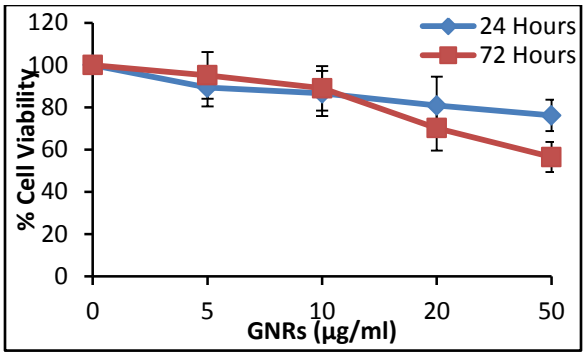

Fig. 4. MTT Assay of HEp-2 cells at 24 and 72 hour after GNR treatment.

Trypan Blue Cell Exclusion Assay showed cell viabilities ranging from 90% at 5µg/mL to approximately 55% at 50µg/mL in 72 hours. (Fig. 5). It was inferred that the incubation of GNRs had led to marked decrease in viable cells. These observations were in concordance with the microscopic appearance of cells as shown in Fig. 3. The experiments were carried out in triplicates.

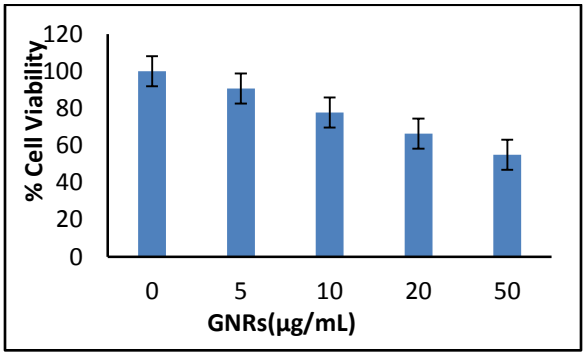

Fig. 5. Trypan Blue Cell Exclusion Assay after 72 hr GNRs incubation.

3.4. Cellular Uptake of Nanoparticles

The cellular uptakes of GNRs by HEp-2cells were studied by conjugating GNR with FITC. GNRs (20µg/mL) were found to have localized inside the cell, including the nucleus, after 4 hours. At 24 hours, GNRs and their location were more visible by immunofluorescence microscopy (Fig. 6).

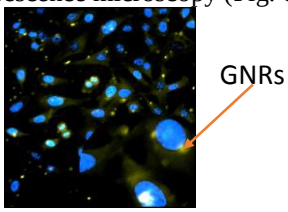

Fig. 6. FITC-Conjugated GNRs in HEp-2 cells (20 µg/mL) 24-hour incubation.

3.5. Gene Expression Studies

Based on cell viability, GNR concentration for HEp-2 cells was set at 5µg/mL and incubated for 4 and 24 hour time intervals. miScript miRNA PCR Arrays was performed using real time PCR. q-PCR analysis showed that of the 86 miRNAs examined, twenty seven showed an increased expression of more than two-fold in 4 hours and 77 miRNAs showed an increased expression of 1.5 to 2-fold in GNR treated cells as compared to control in 24 hours. (Fig. 7).

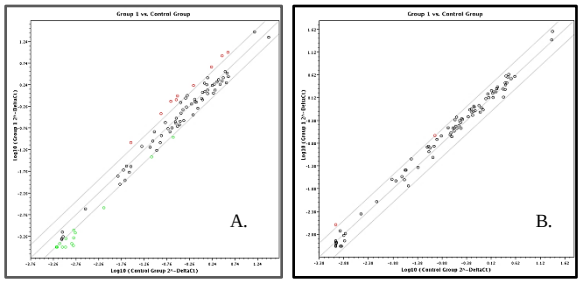

Fig. 7. Scatterplot of Gene regulation after exposure of HEp2 cells with GNRs for (A) 4 Hours and (B) 24 hours.

Specifically, after 4 hr exposure to GNRs the expression of hsa-miR-32-5p with putative target genes: PAXIP1, MDM2, GOLGA3, SIK1) and hsa-miR-7-5p with putative target genes SRSF1, ILF3, C5ORF22, POLR2E, IRS2, PLEC, MEPCE, TRIM47) were upregulated (Fig. 8).

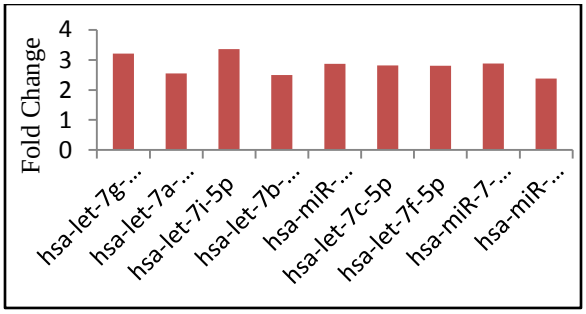

Fig. 8. Gene upregulation after 4 hours exposure with GNRs.

A majority of miRNA were also downregulated after 24 hr. treatment of GNRs in HEp2 cells. These included hsa-miR-142-3p with putative target genes STAM, TGFBR and IRAK1 and hsa-miR-146a-5p with gene IRAK1 (Fig. 9). mRNA target prediction of these miRNAs showed that they may function in inflammation-related signaling.

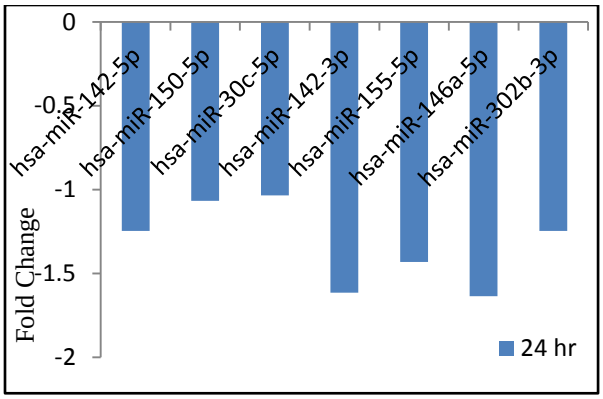

Fig. 9. Gene downregulation after 24 hours exposure with GNRs.

In this study, we identified several miRNAs that are both upregulated and downregulated suggesting that HEp2 cells elicited a response in relation to the added GNRs (Fig. 10).

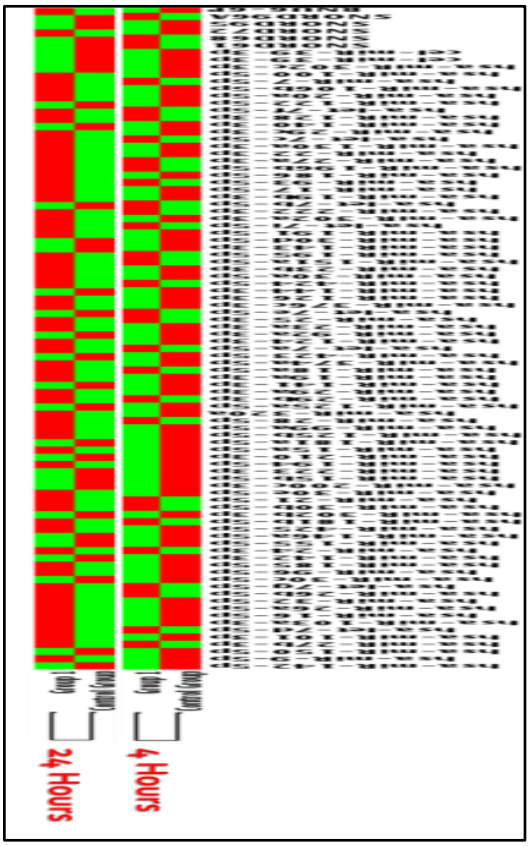

Fig. 10. Heat map showing scaled expression of miRNAs displaying magnitude changes significantly different between the time points (4 Hrs. and 24 Hrs.). The colour scale illustrates the relative expression levels of microRNA across all samples.

CONCLUSIONS

In this study, GNRs were investigated for their toxicity, cell uptake and gene expression analysis. The conjugation of FITC to GNRs was confirmed using FT-IR and zeta potential analysis. GNRs localization was observed inside the cell and even into nucleus after 24hr incubation. Our results shows that the GNRs are non-toxic to human cells, and gene expression evaluation confirmed both up and down regulation of miRNAs showing a correlation between cellular response and the presence of the GNRs in the intracellular environment through inflammation-related signaling.

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