Nanotoxicity Assessment toward the Applications of Carbon Nanotubes as a Small Biomolecule Carrier in Drug Delivery Systems

R.Sharma[*], S.Kwon[**], R. Baktur[**], and A.Sharma[***]

[*]Center of Nanomagnetics and Biotechnology, Florida State University, Tallahassee, FL 32310, USA
[**]Department of Biological Engineering, Utah State University, Logan, UT 84322, USA
[***]Nanotechnology Lab, Department of Electrical Engineering, Maharana Pratap A&T University, Udaipur, Rajasthan 457845, India

ABSTRACT

Carbon Nanotube (CNT) materials has superior properties in electric current carrying capacity, thermal conductivity, and thermal stability. Due to their structure with high aspect ratio, they have structural unusual toxicity and complicate safety issue in a target tissue. In optimized quantities with limited functionality, special type of CNT assembly such as "buckyball" can be used as a potential drug carrier of bioactive molecules and display with increased circulating time and acceptable functionality. We analyzed cytoxicity and inflammatory response following exposure of CNTs. Slow damaging effects of CNT to epidermis and dermis of rat skin was shown using micro-imaging. Physiological perturbation of lung barrier function was observed by measuring transepithelial electrical resistance (TER) during the exposure of different concentrations of CNTs to human lung epithelial cell monolayers in the presence of fibroblast-embedded collagen. The mechanisms of CNTs' toxicity may be closely related to their structure, functional group, and surface charge on the molecule. Further studies are required to probe the mechanisms of cytotoxic and inflammatory responses. We also established the nanoscale toxicity of fullerenes of CNTs.

Keywords: nanotoxicity, carbon nanotube, fullerene, drug carrier

1 INTRODUCTION

Carbon nanotube material has become state of art since it was found biocompatible nanoscale source of delivery carrier in the body. Initially it was considered as cytotoxic and DNA mutant but in last 5 years it was investigated and its inert properties were capitalized as tiny nanomissiles hitting the target and releasing drugs at tissue site very precisely. The electron microscopic structure suggested that drug binding with CNT is not blocking any drug active group and it also remains unidentified against macrophage and immune defense system in the body. Recently, nanobioscience group at Rice University and other institutions established that CNT may be used as safe drug carriers in the body. However, the pitfalls of fullerene structures are that they have high binding and activation energies with likely possibility of binding with circulating free molecules such as hormones, enzymes, peptides and ions. These issues still make the CNT as suspects. We established the CNT molecules transporting across the skin layers with time using microimaging techniques. It showed clearly that epidermis layer of skin is prime target of CNT and CNT can affect the viable skin cells while they are used as drug carriers. Our other direction of CNT effect on live alveolar cells was to observe the inflammatory changes in cells cultured. The synergy of cytokines, nitric oxide production and cytotoxicity of alveolar cells were the main alterations caused during CNT exposure to alveolar cells. The transepithelial electrical resistance of alveolar cells is a unique index identified as CNT induced cytotoxicity biomarker without changing drug delivery properties.

The fullerenes are main CNT constituents. They are bound with drugs at their hydrophilic –C-COO- or –NH- or –SH sites. The pH, temperature, concentration and charge of drug molecules in blood are main factors of rate of delivery.

2 MATERIALS AND METHODS

2.1 Cell Culture

Human bronchial epithelial cells (passage 2-3) (RTTC; Collaborative, Bedford, MA) were used for inflammatory and cytotoxic responses). Cells were seeded (1.5 x 10 cells/cm^2) on top of the polyester membrane attached to fibroblast embedded collagen layers in transwell [1].

2.2 Cytotoxicity

The MTT assay (Sigma) was used to evaluate the changes in cellular metabolic (mitochondrial) activity of cells as a cytotoxic response. Cells were exposed to varying concentrations of SWCNTs. After 48 hours, 150 μL of MTT (5 mg/ml) was added to each well and incubated for 4 hours. Afterward, 850 μL of the MTT solubilization solution (10% Triton X-100 in 0.1 N HCl in anhydrous isopropanol) was added to each well. The resulting formazan crystals was solubilized in acidic isopropanol and quantified by measuring absorbance at 570 nm. Data were

calibrated to the appropriate calibration curve as stated in Sigma protocols [1].

2.3 Inflammatory Responses

Nitric oxide (NO) is produced by many cells in the body. Under normal (basal) conditions, NO is continually being produced by cNOS (constitutive nitric oxide synthase). However, during inflammation, the amount of NO produced by iNOS may be a 1000-fold greater than that produced by cNOS. NO production was measured to identify the level of inflammation (Clancy, Amin, and Abramson 1998). All media samples were analyzed using Griess Reagent system (Promega Corporation, WI) to detect the level of nitrite (NO^{2-}), one of the two stable oxidized products of NO in a liquid phase [1].

2.4 Magnetic Resonance Imaging of Rat Skin Tissues

High resolution 3D FLASH T1 weighted MRI was performed in a 21.1T scanner using a Rf birdcage R 15/900 coil (Bruker Biospin) and PARAVISION 3.2 software at NHMFL. The MRI microimaging was performed before and after placing 10 nm CNT in glass capillaries at different intervals of 2, 4, 6 hours using scan parameters: TR/TE/flip angle = 750ms/ 4.18ms/25°, FOV/matrix size/spatial resolution = 2.6×3.4 cm/ 256×256/0.015 mm, and the inversion time (TI approximately 250 ms) set to null normal skin. Epidermis and hair follicle were measured [2].

2.5 Measurement of Transepithelial Electrical Resistance (TER)

Human bronchial epithelial cells were grown at the interface of air and liquid. Culture media was provided from the bottom through the porous membrane. TER of human bronchial epithelial cell with fibroblasts-embedded collagen layers cultured in Transwell™ was monitored using a portable Voltohmmeter (Millipore, Bedford, MA) attached to a dual "chopstick" or transcellular resistance measurement chamber (Millipore, Bedford, MA). Different concentrations of CNTs were exposed to the co-culture layers for 6 hours. Each of the two electrode systems contained Ag/AgCl electrode for measuring voltage and a concentric spiral of silver wire for passing current across the epithelium. Electric current could then be passed across the epithelium to measure TER (ohms.cm^2). It is perceived that TER values higher than the background fluid resistance indicate a confluent airway epithelium with tight junctions. TER was monitored to identify the perturbation in the normal physiology and permeability of human bronchial epithelial cells [1].

2.6 Drug metabolizing enzymes

The leucine aminopeptidases, hepatic lysosomal enzymes, esterases were main drug metabolizing enzymes. The measurement of enzymes was described elsewhere [3].

3 RESULTS

Inflammatory and Cytotoxic Responses: Nitric oxide (NO) production following exposure of single walled carbon nanotubes (SWCNTs) to epithelial cells was dramatically increased as the concentration of SWCNTs increased (Figure 1A). At higher concentrations of SWCNTs, cells showed cytotoxic response and parts of cell layers were detached (data not shown). Each NO production was normalized by total proteins. Cellular metabolic activity was observed following exposure of different concentrations of SWCNTs to both cell layers. MTT activity was decreased as concentration of SWCNTs increased, especially for epithelial cells (Figure 1B) [1].

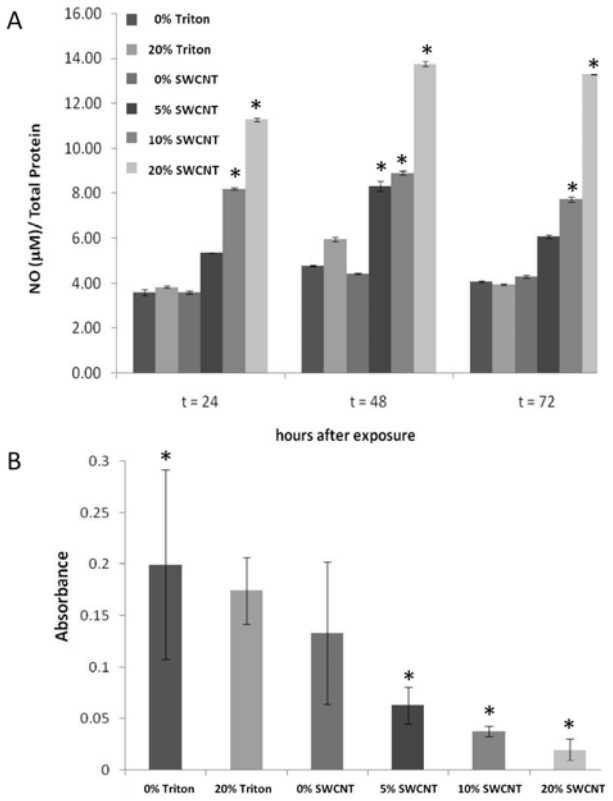

Figure1. Effect of SWCNTs on NO production and cell viability from epithelial cell layers. NO production following exposure of SWCNTs to epithelial cells was dramatically increased as the concentration of SWCNTs increased (A). At higher concentrations of SWCNTs, cells showed cytotoxic response (B). Each NO production was normalized by total proteins. MTT assay was used to show cytotoxic response. * denotes a significant difference from the control (0% SWCNT) ($p < 0.05$). Results were

NSTI-Nanotech 2009, www.nsti.org, ISBN 978-1-4398-1783-4 Vol. 2, 2009

presented as mean ± SD. Number of replicates for (A) was four. Number of replicates for (B) was sixteen.

The level of NO production was different following different time of exposure of SWCNTs. In particular, effects of exposure time on NO production was more significant in the presence of serum in the media during exposure. Also, it was observed that lung epithelial cells uptake more SWCNTs in serum-containing media than in serum-free media.

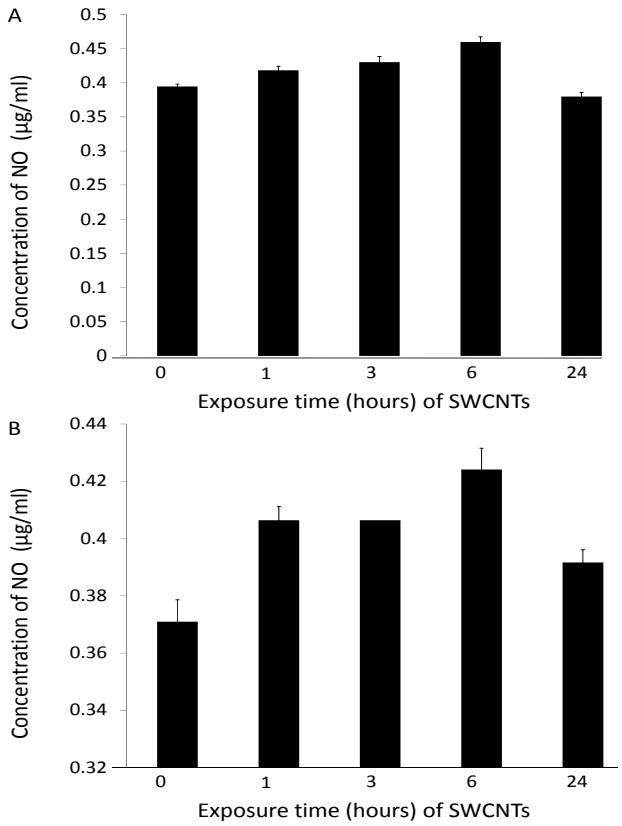

Figure2. Effect of exposure time of SWCNTs on inflammatory response in lung epithelial cells in serum-free media (A) and in serum-containing media (B). Concentration of SWCNTs was 4mg/L.

Microimaging of Rat Skin Tissues: Cellular Damage at CNT-Skin Tissue Interface: The ex vivo MRI 3D FLASH images showed axial, sagittal and coronal images. The coronal images are shown in Figure 2. The images showed distinct morphological and structural features of 3 skin layers. The ex vivo excised skin MRI of the ventral abdomen skin showed epidermis, dermis, hair follicle, sebaceous oil gland as shown in Figure 2. The CNT-skin interface showed consistent damage to skin tissue on MRI microimages shown by arrows in Figure 2. The hair follicles remained intact while epidermis membrane and dermis vasculature was badly damaged. The skin features were distinct and measurable. The dimentions of skin layers were measured as epidermis (150-200 micrometers; hair root(300 micrometers); hair follicle(50 micrometers); dermis(600-650 micrometers. The 35 nanometer CNT as drug carrier passed through epidermis in 2 hours and whole dermis in 6-8 hours.

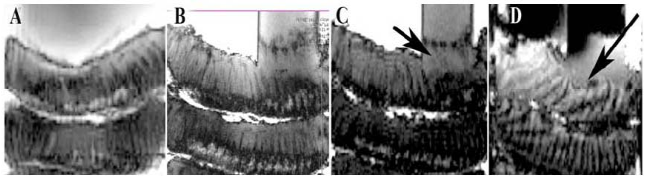

Figure 2: The skin microimaging at 21 Tesla MRI using fast 3D FLASH technique shows control without carbon nanotubes (A); carbon nanotube sample in tube placed for 3 minutes on skin top (B); after 15 minutes carbon nanotube sample in tube placed on skin top (C); after 6 hours carbon nanotube sample stayed on top of the skin (D). Notice the slow damage to epidermis and dermis by carbon nanotubes caused shown by arrow.

Effect of CNTs on Physiological Function of Airway Epithelial Cells: Different concentrations of SWCNTs were exposed to the co-culture layers for 6 hours. The TER of the controls (5% and 20% of Triton X-100 and 0% of SWCNT) were stable around 500 ohms.cm^2 (resistance of epithelial-free tissue was subtracted) for 48 hours. 10-20% of SWCNTs rapidly compromised the barrier function of the epithelium and the TER decreased to 120 ohms.cm^2. After removing SWCNTs, the TER completely recovered to the control level (Figure 3) [1].

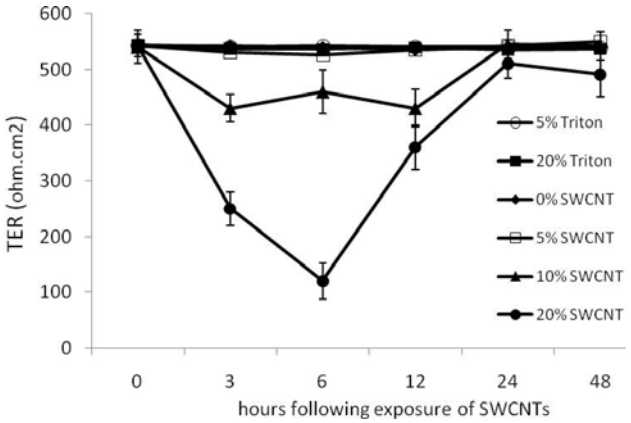

Figure 3. Exposure of SWCNTs to co-culture layers impacts transepithelial electrical resistance (TER). Human bronchial epithelial cells were grown at the interface of air and liquid. Culture media was provided from the bottom through the porous membrane. TER of human bronchial epithelial cell with fibroblasts-embedded collagen layers cultured in Transwell™ was monitored using a portable Voltohmmeter (Millipore, Bedford, MA) attached to a dual "chopstick" or transcellular resistance measurement chamber (Millipore, Bedford, MA). Different

concentrations of SWCNTs were exposed to the co-culture layers for 6 hours. The TER of the controls (5% and 20% of Triton X-100 and 0% of SWCNT) were stable around 500 ohms.cm^2 (resistance of epithelial-free tissue was subtracted) for 48 hours. 10-20% of SWCNTs rapidly compromised the barrier function of the epithelium and the TER decreased to120 ohms.cm^2. After removing SWCNTs, the TER completely recovered to the control level.

4 DISCUSSION

The CNT molecule size is very important as we established that the skin epidermis layer 150-175 microns thick is combined layers of viable cells. The CNT size between 35-100 nanometers seems suitable to pass across the skin epidermis barrier. The rate of diffusion and transport is also a size dependent criterion to evaluate the CNT material as biocompatible drug carrier. Typically the skin cell membrane made of phospholipids, cholesterol and lipoproteins plays active role in controlling the ion transport inside outside across the channels or ion pores using active energy against concentration gradient. The CNT molecules had very small size 35 nanometers in our experiment so we believe that drug molecule (105-200 angstrong) size attached with insert CNT does not expose its hydrophilic bonds so inert CNT molecules keeps bound drug molecule safe while passing across the membrane and later releases it at suitable pH to act at the tissue target site.

The fullerenes constitute major components of CNT material. They cause generation of cytotoxic anion species. The major mechanisms are believed to support CNT induced toxicity are following: 1.Fullerenes of nanotubes showed free radical chemistry, attraction to electrons, antioxidant properties; 2. Some Carbon-60 fullerenes bind to nucleotides, hamper self-repair in double–strand DNA; 3. CNT display high electrical and thermal conductivity, high strength, rigidity. Medical/nonmedical applications suggest occupational, accidental exposure; 4. Fullerens(cages), single wall nanotubes, multi-walled nanotubes show toxicity. CNT produce superoxide anion, lipid peroxidation, cytotoxicity in plants and animals; 5. Uncoated fullerenes in *largemouth bass* fish showed lipid peroxidation in brain tissue and glutathione depletion in gills. 6. C60 toxicity increases by Poly Vinyl Propylene due to stable charge transfer complexes. 7. Metal catalysts like THF may pass through blood-brain barrier, commonly used in nanotube fabrication; 8. More derivatized fullerenes are less toxic, due to low efficiency in ROS generation; 9. CNT showed toxicity effects, dose dependent experimental epitheloid granuloma; 10. At optimized CNT single walled CNT concentrations, low Taxotere quantities encaged inside may target breast tumor tissue more efficiently; 11. Cultured alveolar fibroblasts following exposure of CNTs showed possibility of transplanting CNT encaged fibroblasts. In conclusion, medical/nonmedical drug delivery system applications of CNT suggest its use with care due to occupational, accidental exposure and nanotoxicity as health concern.

Other side story of CNT based drug delivery systems is successful drug transport, delivery and release without any change or deactivation of hepatic drug metabolizing enzymes (data not shown). Typically drug metabolizing esterases, peptidases, NADPH oxidases, diphorases biotransform the drug into bioactive metabolites to make them either compete with natural enzymes or intermediary metabolic pathway(s) or act as effectors (stimulators or inhibitors) at certain biochemical metabolic step. Fullerene molecules in CNT are found not to participate in such metabolic enzyme reactions while slowing they are excreted out unnoticed leaving behind the drug at site.

One of the major concerns regarding the potential risks of nanoparticles is their capacity to penetrate cells and potentially translocate to other cells, tissues and organs remote from the portal of entry to the body. This is considered to be a necessary step in the movement of particles deposited in the lung, entering the blood, acting upon cells in other tissues, manifesting ultimately in a physiological response. The mechanisms of translocation across the respiratory epithelium, and the resulting possible toxic effects in and beyond the lung have not been well characterized yet. Further investigation need to be focused on achieving the folwing outcomes: 1. Identifying which features of nano-particles/tubes/fibres are important in particle-cell interactions, considering the potential role of nanoparticle chemistry, structure, mass, numbers, shape, surface area, surface charge and surface functionalisation; 2. Suggesting how nanoparticles may be modified to enhance or reduce their capacity to enter cells; 3. Suggesting how interactions between nanoparticles and cultured human cells might be studied.

Due to the nature of nanoparticles (in particular, nano-scalesize and agglomeration), nanoparticles may penetrate without any surface reaction to enhance or inhibit inflammatory protein expression. Agglomerated nanopartices (micro-scale) may induce inflammation through the surface receptor-mediated process. Functionalized nanoparticle may undergo different process to affect inflammatory and cytotoxic responses. Further studies are required to probe the mechanisms of cytotoxic and inflammatory responses.

REFERENCES

[1] Stoker et al. *International Journal of Toxicolgy* (2008)

[2] Sharma (2008) http://dx.doi.org/10.1016/j.mri.2008.06.013

[3] Sharma R.2008 Nature Precedings. **precedings**.**nature**.com/documents/2738/version/1/files/npre20082738-1.pdf

[4] Michael Berger. January 22, 2009 Nanowerk