Curcumin coating: A Novel Solution to Mitigate Carbon Nanotube Toxicity

M.Sc. Thesis

By

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree

of

Master of Science

by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"Curcumin coating: A Novel Solution to Mitigate Carbon Nanotube Toxicity"** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE in Biotechnology** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from August 2021 to May 2023 under the supervision of Dr. Hem Chandra Jha, Associate professor, Indian Institute of Technology Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

SRele.

(Samiksha Rele)

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

16/5/2023

Signature of the Supervisor of M.Sc. thesis
(Dr. Hem Chandra Jha)

Samiksha Rele has successfully given her M.Sc. Oral Examination held on **8th May 2023.**

16/5/2023

Signature of Supervisor of MSc thesis
Date:

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Date:

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Signature of PSPC Member
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Date:

Sharad Gupta 16/05/2023

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(Dr. Sharad Gupta)
Date:

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"Success is not achieved by the work of one but with the help of many"

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Lastly, and most importantly, I extend my deepest appreciation towards my **family** for their unwavering support, encouragement, love, care and blessings.

Thank you!

Samiksha Rele

DEDICATION

This thesis is dedicated to my family.

Their love and support made me who I am today.

ABSTRACT

Multi-walled Carbon Nanotubes (MWCNTs) are inert and stable structures made by rolling of graphene sheets into concentric cylinders. They have high surface area to volume ratio thereby provide greater space for attachment of drug molecules. MWCNTs are being widely researched upon as agents for tissue engineering, in-vivo imaging and vehicles for targeted drug delivery in cancer and many other diseases. These are largely non-toxic in nature however, when cells are exposed to these nanotubes for prolonged durations or at high concentrations, they show some adverse effects. These include cytotoxicity, inflammation, generation of oxidative stress, and genotoxicity among others. To combat such adverse effects, various moieties can be attached to the surface of these nanotubes. Curcumin is a known anti-inflammatory, antioxidant and cytoprotective compound derived from a medicinal plant called *Curcuma longa*. In this study, we have evaluated the anti-inflammatory, antioxidant and antiapoptotic effect of Curcumin coating on the surface of MWCNTs. The results show a significant decrease in the level of pro-inflammatory molecules in Curcumin-coated MWCNT treated cells compared to uncoated ones. Further, compared to the uncoated, there is a reduction in oxidative stress, mitochondrial damage and apoptosis in the cells treated with Curcumin-coated MWCNT. Our findings suggest that coating of Curcumin on the surface of MWCNTs reduces its ability to cause inflammation, oxidative stress and apoptosis.

LIST OF PUBLICATIONS

Manuscript submitted and under review

"Comprehensive insight into altered host cell-signalling cascades upon Helicobacter pylori and Epstein Barr virus infections" *Kashyap D; **Rele S**; Bagde P; Saini V; Chatterjee D; Jain A; Pandey R; Jha H C.*

Archives of Microbiology, April 2023 **(under minor revision)**

Manuscript under preparation

"Curcumin coating on the surface of multi-walled carbon nanotubes mitigates inflammation, oxidative stress and apoptosis caused by their exposure" ***Rele S;*** *Thakur C; Baral B; Moorthy H N; Jha H C.*

"Anti-inflammatory and ROS scavenging activity of *Cannabis sativa* leaf extract in bacterial and viral exposed lung and neural cells." *Baral B, Saini V, **Rele S**, Tambat N, Singh S, Tandon A, Tambat P and Jha H. C.*

"SARS-CoV-2 Envelope protein induces necroptosis and mediates inflammatory response in lung and colon cells through Receptor Interacting Kinase 1" *Baral B; Saini V; Kundu P; Tandon A; Singh S; **Rele S** and Jha H. C.*

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ACRONYMS

MWCNT	Multi-walled Carbon Nanotube
SWCNT	Single-walled Carbon Nanotube
CNT	Carbon Nanotube
NFκB	Nuclear Factor Kappa B
TNFα	Tumor Necrosis Factor α
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-1β	Interleukin 1-β
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
WB	Western Blot
Lys40	Uncoated Lysine functionalised MWCNTs (40μg/ml)
Lys20	Uncoated Lysine functionalised MWCNTs (20μg/ml)
Cur40	Curcumin coated Lysine functionalised MWCNTs (40μg/ml)
Cur20	Curcumin coated Lysine functionalised MWCNTs (20μg/ml)
DMSO	Dimethyl Sulfoxide
EB	Ethidium Bromide
AO	Acridine Orange
DCFDA	2'-7'-Dichlorodihydrofluorescein diacetate
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
ROS	Reactive Oxygen Species

Chapter 1: INTRODUCTION

1.1. NANOTECHNOLOGY

Nanotechnology is an ever-expanding field of science that deals with creating, and manipulating materials on a nanoscale level. It offers a unique opportunity to utilize the properties of materials at the nanoscale to develop novel technologies and solutions to a diverse range of scientific and engineering challenges. Nanotechnology has the potential to revolutionize many fields, including medicine, electronics, energy, and environmental sciences.

At the nanoscale, materials exhibit extraordinary properties that differ significantly from their bulk counterparts [1], making it possible to design and fabricate new materials with specific properties. The applications of nanotechnology are numerous and varied. It offers tremendous potential for developing medical diagnostics [2] and new drug delivery systems for cancer [3], [4], and many other diseases [5].

1.2. CARBON NANOTUBES AND THEIR APPLICATIONS

Carbon nanotubes are cylindrical structures made of carbon atoms that exhibit unique mechanical, electrical, and thermal properties. Due to their remarkable properties, carbon nanotubes have gained attention as promising nanomaterial for use in biology research and medicine [6].

They are primarily divided into two subtypes – Single-walled carbon nanotubes (SWCNTs) and Multi-walled carbon nanotubes (MWCNTs).

Multi-walled Carbon Nanotubes (MWCNTs) are made by rolling of sp2 carbon containing multiple graphene sheets into concentric cylinders. Their diameter ranges from 7 to 100 nm and length can vary up to a few hundred nanometers[7]. They are chemically inert in nature and have high surface area to volume ratio which makes them a suitable candidate for drug loading or attachment.

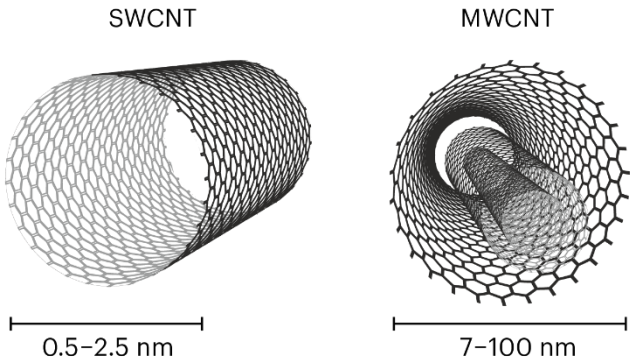


Figure 1. Single-walled Carbon Nanotube (SWCNT) and Multi-walled Carbon Nanotube (MWCNT) [8]

In medicine, carbon nanotubes have shown great potential for drug delivery and imaging applications. Carbon nanotubes can be used as carriers for drugs or therapeutic agents, allowing for targeted delivery to specific cells or tissues [8]. The high aspect ratio and surface area of carbon nanotubes can enhance the delivery efficiency of therapeutics, making them a promising candidate for use in tissue engineering [9] and targeted drug delivery. Carbon nanotubes have been used as contrast agents for imaging techniques such as MRI and CT scans, providing enhanced resolution and sensitivity compared to traditional contrast agents [10].

Furthermore, carbon nanotubes have been explored for their antibacterial properties [11]. Studies have shown that carbon nanotubes can inhibit the growth of bacteria and biofilms, making them a potential candidate for use in wound dressings and other medical applications [12].

Carbon nanotubes have gained significant attention in the field of tissue engineering due to their unique mechanical and biocompatible properties. They have shown potential for use as scaffolds, delivery vehicles, and substrates in tissue engineering applications.

In conclusion, carbon nanotubes are a promising nanomaterial with potential applications in biology research and medicine. Their unique properties and versatility make them an attractive candidate for use in a wide range of biomedical applications.

1.3 POTENTIAL HEALTH HAZARDS

While carbon nanotubes have shown great promise in various fields, including medicine and biology research, there are also potential health hazards associated with their use. The small size and unique properties of carbon nanotubes can lead to unintended biological effects, and studies have raised concerns about their potential toxicity.

1.3.1 Cytotoxicity

Several studies have reported that CNTs can induce cytotoxic effects in various in-vitro and in-vivo models [12]. The mechanism of CNT-induced cytotoxicity is multifactorial and depends on various factors, including the length, size, surface area, surface chemistry, and shape of the nanotubes [13], [14], as well as the cell type and exposure conditions [15].

Carbon nanotubes can also accumulate in various organs, including the liver, spleen, and kidneys, which can lead to organ damage and toxicity [16]. There are also concerns about the long-term effects of carbon nanotube exposure, as chronic exposure may increase the risk of developing cancer [17] or other chronic diseases.

To mitigate the cytotoxicity of carbon nanotubes, researchers are exploring various strategies, including the use of protective coatings, functionalization with biocompatible molecules, and the engineering of carbon nanotube structures to reduce their toxicity.

1.3.2. Inflammation

Inhalation of carbon nanotubes is a particular concern, as it can lead to lung damage and respiratory problems. Studies have shown that carbon nanotubes can induce inflammation and fibrosis in the lungs [18], which can lead to chronic lung disease [19]. Several studies have shown that CNTs can induce inflammation in various organs and tissues, including the lungs, liver, spleen, and brain [20]. The mechanisms of CNT-induced inflammation involve the activation of immune cells, such as macrophages, and the release of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) [21], and tumor necrosis factor-alpha (TNF-α). Furthermore, CNTs can also activate the inflammasome, a multiprotein complex that regulates the secretion of IL-1β, resulting in the amplification of the inflammatory response. The physical properties of CNTs, such as their high aspect ratio and needle-like shape, can contribute to their inflammatory potential by inducing the formation of reactive oxygen species (ROS), which can lead to oxidative stress and subsequent inflammation.

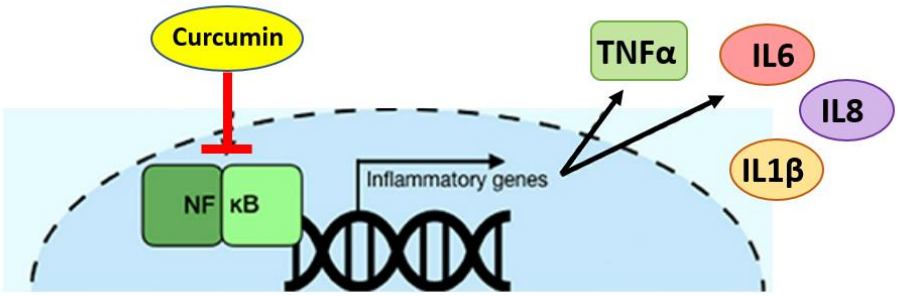


Figure 2. Curcumin inhibits NFκB which later inhibits the translation of pro-inflammatory cytokines like IL-6, IL-8, IL-1β and TNFα. [21]

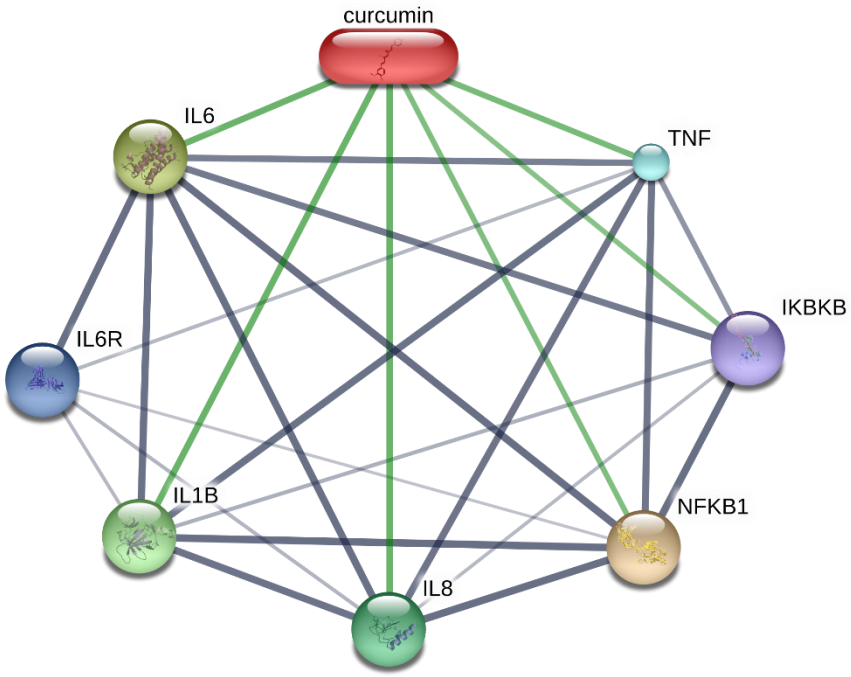


Figure 3. Connectome showing interaction between Curcumin and Inflammatory markers (made using stitch.embl.de)

1.3.3. Oxidative stress

Oxidative stress is a common mechanism of toxicity associated with carbon nanotubes (CNTs). The generation of reactive oxygen species (ROS) is a major contributor to oxidative stress, and several studies have shown that CNTs can induce ROS production in various cell types and tissues [22]. Additionally, carbon nanotubes have been found to penetrate cell membranes and interact with cellular components, which can lead to oxidative stress [23] and DNA damage [24].

1.3.4. Apoptosis

Several studies have shown that CNTs can induce programmed cell death or apoptosis in different cell lines upon prolonged exposures. CNTs enter the cells via endocytosis [25] or through direct piercing [26]. Such actions lead to the induction of inflammation and oxidative stress due to ROS imbalance. All these happenings eventually lead to activation of the apoptotic pathways which causes cell death [27].

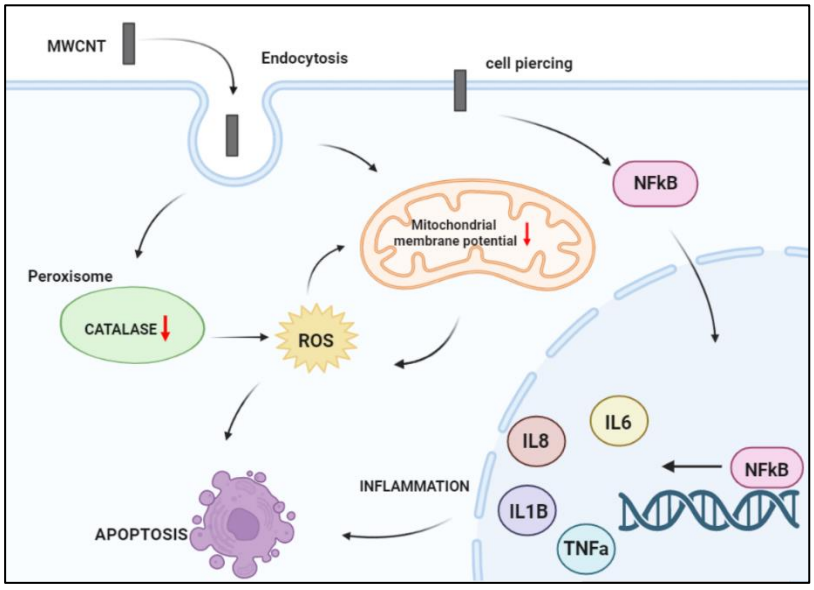


Figure 4. Mechanism of action of CNTs that leads to inflammation, oxidative stress and apoptosis

1.4. CURCUMIN

Curcumin is the major bioactive ingredient of the medicinal plant Curcuma longa. It is a natural, plant-based compound with a molecular weight of 368.4 Da and molecular formula $C_{21}H_{20}O_6$.

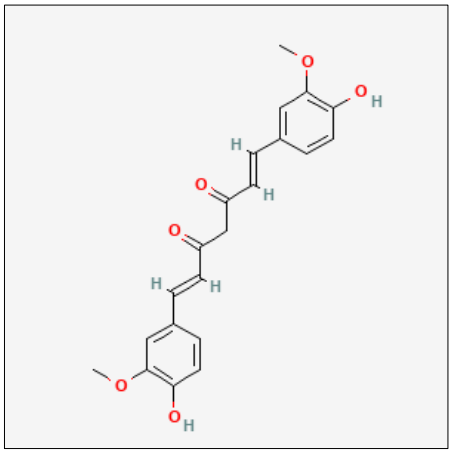


Figure 5. 2-D structure of Curcumin
PubChem ID – 969516

1.4.1. Anti-inflammatory effects of Curcumin

Since ancient times Curcumin is known to show anti-inflammatory effects. Its anti-inflammatory activity has been well established on different cell lines moreover, its most prominently seen on Lung carcinoma cell line (A549) [28]. Curcumin inhibits NFκB, which is the central mediator of inflammation [29]. NFκB is a transcription factor that promotes the expression of pro-inflammatory proteins like TNFα and pro-inflammatory cytokines like IL-6, IL-8, IL-1β, etc. Hence, it has been observed that Curcumin reduces the expression of several major inflammatory proteins, cytokines and chemokines [30].

1.4.2. Antioxidant activity of Curcumin

Curcumin also has well established antioxidant properties. Oxidative stress is a condition that arises due to an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms. It can contribute to the development and progression of various diseases, including cancer, cardiovascular disease, neurodegenerative disorders, and diabetes.

Curcumin's antioxidant effects are mediated by several mechanisms, including the scavenging of ROS [31], inhibition of lipid peroxidation, and upregulation of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [32]. Additionally, Curcumin has been shown to increase the activity of other important cellular antioxidant defense mechanisms, such as the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [33].

1.4.3. Cytoprotective nature of Curcumin

Cytoprotection refers to the ability of a substance to protect cells from various stressors, including oxidative stress, inflammation, and toxins. Curcumin has been shown to exert cytoprotective effects through multiple mechanisms, including antioxidant, anti-inflammatory, and antiapoptotic activities. Curcumin modulates various signaling pathways involved in cell survival and death, including the PI3K/Akt, MAPK, and NF-kB pathways [34].

1.5. ADVANTAGES OF SURFACE-COATED MWCNTS

MWCNTs have been reported to show several adverse effects on cells like cytotoxicity, inflammation, oxidative stress, etc. Surface modification is an

emerging field in nanotechnology which aims towards modifying the surface functional groups of nanomaterials in such a way that it mitigates their adverse effects. Moreover, studies have shown that surface modification also helps in increasing the solubility of CNTs [35]. Researchers around the globe are trying to find biocompatible compounds which can be coated onto such nanomaterials to enhance their properties. Curcumin is a potential candidate molecule for such endeavors as it does not harm the cells and at the same time mitigates the inflammatory, cytotoxic and oxidative stress generation activity of MWCNTs.

Chapter 2: AIM AND OBJECTIVES

2.1. AIM

The aim of our study is to check if Curcumin coating on the surface of Multi-walled Carbon Nanotubes (MWCNTs) is able to mitigate their inflammatory, oxidative stress generating and apoptosis inducing effects in-vitro using human Lung epithelial cell line (A549).

2.2. OBJECTIVES

This study can be divided into three major objectives: -

2.2.1. To check the anti-inflammatory effect of Curcumin coating on MWCNTs

2.2.2. To check the antioxidant effect of Curcumin coating on MWCNTs

2.2.3. To check the antiapoptotic effect of Curcumin coating on MWCNTs

2.3. STUDY DESIGN

In this study, we have used two drugs. One is a Lysine-functionalised Multi-walled Carbon nanotube, denoted as Lys_ throughout the thesis and other is Curcumin-coated-lysine-functionalised Multi-walled Carbon nanotube, denoted as Cur_ throughout the thesis.

We have chosen two concentrations of these drugs throughout the study, namely, 40μg/ml and 20μg/ml. Hence the groups are denoted as follows: -

Lys40: Uncoated Lysine functionalised MWCNTs (40μg/ml).

Lys20: Uncoated Lysine functionalised MWCNTs (20μg/ml).

Cur40: Curcumin coated Lysine functionalised MWCNTs (40μg/ml).

Cur20: Curcumin coated Lysine functionalised MWCNTs (20μg/ml)

Chapter 3: MATERIALS AND METHODS

3.1. Materials

3.1.1. Instruments and equipment

Pipettes, tips, tissue culture plates, 15ml and 50ml falcon tubes, microcentrifuge tubes (MCTs) Laminar Air Flow (LAF), Bath sonicator, refrigerators, autoclave, microplate reader, centrifuge, vortex, spinner, Gel Doc, PCR, Nano-drop, fluorescence microscope, cell culture hood, water bath, heat block. SDS-PAGE unit, CO_2 incubator, distilled water unit, pH meter, rocker.

3.1.2. Reagents

Phosphate buffer saline (PBS), MQ water, distilled water, Dulbecco's modified Eagle's medium (DMEM), trypsin, fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO), antibiotics, ethanol, chloroform, isopropanol, Glycine, Tris-buffer, tween-20, antibodies (primary and secondary), ECL, SyBr green, Mitotracker Red and Green, Ethidium bromide, Acridine orange, DCFDA dye, methanol, TRIzol, primescript Takara cDNA synthesis kit, RIPA buffer, protease inhibitor, phosphatase inhibitor, BSA (Bovine Serum Albumin), PMSF, protein loading dye, protein ladder, MTT dye, APS, TEMED, Ponceau stain.

3.1.3. Drugs/Compounds

Lysine-functionalized multi-walled carbon nanotubes and Curcumin-coated lysine-functionalized multi-walled carbon nanotubes were received from Prof. Hari Narayan Moorthy, Department of Pharmacy, Indira Gandhi National Tribal University, Amarkantak, Madhya Pradesh.

3.2. Cell Culture

The Human Epithelial Lung carcinoma cell line, A549, was obtained from the National Center for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Himedia, Mumbai, India) containing 10% fetal bovine serum (FBS; BioWest, South America origin) and 100 U/ml penicillin-streptomycin (Himedia, Mumbai, India) under specific conditions of 5% CO_2 and humidified air at 37°C (Eppendorf India).

3.3. Treatment and Sample preparation

Lysine functionalised MWCNTs and Curcumin coated lysine functionalised MWCNTs were sonicated for 15 minutes at 50°C in bath sonicator. They were then dissolved in cDMEM to achieve a final concentration of 40µg/ml and 20µg/ml. Cell were given 2 PBS washes and then incubated with the required concentration of nanotubes for the desired time period. Post incubation cells were scraped off the tissue culture plate and collected in a 15 ml tube. They were then centrifuged at 2500 rpm for 5 minutes followed by a PBS wash and again centrifugation at 2500 rpm for 5 min. The cell pellet collected was dried and stored at -20 degrees for till further processing.

3.4. Cell survival assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as previously described[36]. A549 cells were seeded in 96-well plate at 50% confluency and incubated for 24 hours. Uncoated and Curcumin coated MWCNTs were diluted to form 6 concentrations via serial dilution namely, 40 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml

and 1.25 µg/ml. Cells were incubated with these dilutions for 24 hours. Post incubation, media was removed and cells were washed with PBS. Then, 50µL of 0.5mg/ml MTT dye was added to each well and cells were incubated in dark for 3 hours at 37°C. After incubation, MTT was removed carefully, and formazan crystals were dissolved by the addition 100 µL DMSO to each well. Cells were then incubated for another 2 hours in dark on an orbital shaker at 220rpm. Absorbance was measured using a microplate reader at 570nm.

3.5. RNA Isolation

The cell pellet was incubated in TRIzol reagent overnight. It was then vortexed and pipetted until the pellet was completely dissolved. 100µl of chloroform was added and incubated at room temperature for 3 minutes. The MCTs were then centrifuged at 12,000g for 15 minutes. The aqueous phase was transferred into a fresh MCT. 250µl of isopropanol was added to each MCT and incubated at room temperature for 10 minutes. They were centrifuged at 12000 g for 15 minutes and the supernatant was discarded. Then 500µl of 75% ethanol was added to each tube and incubated for 5 minutes. The contents were centrifuged at 8000 g for 7.5 minutes and supernatant was discarded. The pellet was air dried for 20 minutes and 20µl of autoclaved water was added to each tube. The quantification of RNA was performed using nano-drop.

3.6. cDNA synthesis

2µg RNA was used to prepare cDNA using TAKARA cDNA synthesis kit. For each sample, 2µl random hexamer, 1µl dNTPs and 10µl RNA template was mixed and incubated at 65°C for 5 minutes. Later, 4µl 5x buffer, 1µl

Reverse transcriptase, 0.5µl RNase inhibitor and 1.5µl autoclaved water was added per sample and PCR reaction was performed.

Stage 1 - 30°C – 10 min

Stage 2 - 42°C – 60 min

Stage 3 - 70°C – 15 min

Stage 4 - 4°C – ∞

3.7. Quantitative real-time PCR

Prepared cDNA was diluted with 40µl autoclaved water per 1µg of cDNA. qRT-PCR was performed using SYBR green real-time master mix (Thermo Scientific, USA) on Agilent AriaMX. Primers were procured from IDT for GAPDH, IL6, IL8, IL1β, NFκB and TNFα. (Annexure-A). The relative gene expression of the target genes was analysed using the 2-ΔΔCt method. Briefly, delta Ct (ΔCt) is the difference obtained after subtracting the cycle threshold (Ct) value of gene of interest and GAPDH. Further, delta delta Ct (ΔΔCt) is the difference between the delta Ct (ΔCt) of the sample (D1/3) after infection/treatment and the control sample (D0). These values we finally used to calculate the fold change.

3.8. Protein Isolation

Cells were seeded in 60mm tissue culture plates at 50% confluency. Treatment with MWCNTs was given at 80% confluency. The cell pellet was collected after 6 hours and 12 hours of incubation. Protein isolation was performed using RIPA (radioimmunoprecipitation assay) lysis buffer. Protease and phosphatase inhibitors were added. 100µL of this mixture was added to all pellets. The tubes were vortexed for 30 seconds every 5 min

over a period of 1 hour. The contents were then transferred to MCTs and centrifuged at 13,000 rpm for 30 minutes. The supernatant was carefully collected in a separate MCT.

3.9. Protein estimation

Total protein in the cell was estimated using Bradford Assay. 150µl of Bradford reagent was mixed with 50µl of protein and water mixture in 96-well plate. Absorbance was recorded at 595nm in duplicates. A BSA standard plot was used to then quantify the amount of protein in each sample.

3.10. SDS-PAGE

A polyacrylamide gel was casted such that it had a 12% Resolving gel and a 5% stacking gel. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed by loading equal amount of total protein in each sample. Separated proteins were transferred onto 0.22µm nitrocellulose membrane.

3.11. Western blot

Membrane blocking was done using 4.5% BSA. Target proteins were identified by incubation with the following primary antibodies for 2 hours in 1:1000 dilution – anti-NF-κB, anti-TNFα, anti-Catalase and anti-GAPDH. After washing, the membrane was incubated for 1 hour in horseradish peroxidase-conjugated secondary antibodies against the respective primary antibodies in 1:3000 dilution. Target proteins were detected using a chemiluminescent ECL Western blotting substrate

(BioRad, Hercules, California, USA.). Image analysis and quantification was performed using Image J software (National Institutes of Health, Bethesda, MA, USA).

3.12. Cellular ROS estimation

Cellular ROS production was measured using DCFDA (2'-7'-Dichlorodihydrofluorescein diacetate) dye[37] 12 hours post incubation with MWCNTs. Briefly, the live cells were stained with 10 μg/mL of the dye in PBS and incubated for 20–25 min, followed by a wash with PBS, and visualization was done under Olympus IX83 fluorescent microscope aided with cell Sens imaging software at 20× objective magnification. The amount of intracellular ROS was proportional to DCF fluorescence intensity and was quantified using ImageJ software. Relative changes in DCF fluorescence were expressed as fold increases over the control cells.

3.13. Mito-tracker Red-Green assay

Mito tracker red- green staining method was used to assess the changes in mitochondria membrane potential as described [37]. Mito tracker red was used to access the active mitochondria and its accumulation is dependent on mitochondrial membrane potential. Meanwhile, Mito tracker green binds to mitochondrial proteins regardless of their membrane potential and represents the mitochondrial mass. 12h post incubation to MWCNTs, cells were treated with (200 nM) of Mito tracker red in 500 μL of plain DMEM incubated for 40 minutes at 37 °C. After incubation cells were washed with PBS followed by treatment of (100 nM) Mito tracker green in 500 μL of serum-free media for 40 min. After completion of the incubation period cells were washed with PBS and images were taken under Olympus IX83

fluorescent microscope aided with cell Sens imaging software at 20× objective magnification.

3.14. Cell death assay

For apoptosis measurement, Ethidium bromide (EB) and Acridine orange (AO) dual fluorescent staining method was used as prescribed [38]. Both dyes were used in a 1:1 ratio, such that the concentration of EB and AO was 100 μg/ml. Cells were seeded in 12-well plate at 50% confluency. After 24 hours they were incubated with the desired concentrations of Curcumin coated and uncoated MWCNTs. Post 48 hours incubation, media was removed, cells were given 2 PBS washes and 100μl of EB/AO dye was added to each well. The plate was incubated in dark at 37°C for 5 min. Again, 2 PBS washes were given. The plate was then monitored using Bright field, TRITC and FITC filters by fluorescence microscopy to observe red, green, orange and yellow cells. The cells were then counted and assigned respective categories and a percentage bar graph was plotted.

3.15. Statistical analysis

Data were statistically analyzed using a two-tailed Student t-test. All the results were derived from a set of duplicate experiments. P values were estimated using GraphPad Prism version 8, and P values of <0.05, <0.01, and <0.001 were considered statistically significant and represented by *, **, and ***, respectively.

Chapter 4: RESULTS AND DISCUSSION

4.1. Curcumin coating on the surface of MWCNTs reduces its cytotoxic effects

From the results of MTT assay it could be observed that Curcumin coated MWCNTs showed a greater cell survival percentage than the uncoated MWCNTs. After 24 hours of incubation **(Fig. 6. a)**, both Curcumin coated and uncoated MWCNTs showed no significant cytotoxicity. The survival percentages in both groups remained above 90%. From this we can infer that it is safe to use 40μg/ml or lower concentration of drug treatment until the time point is less than 24 hours. Also, if we carefully observe, the percentage survival for Curcumin-coated MWCNTs (94%) was slightly more than the uncoated ones (91%). However, after 48 hours of incubation, **(Fig. 6. b)** it was observed that the percentage cell survival in the uncoated MWCNTs (43.2%) was critically lower than the Curcumin-coated ones (66.4%). This indicates that Curcumin exerts a cytoprotective effect which helps in protection of cells against stress and death. Previous reports have been shared in literature showing similar cell survival percentages for MWCNTs and 24 and 48 hours [39]. However, this is the first study that involves the usage of Curcumin coated MWCNTs and hence, no literature is available for the same.

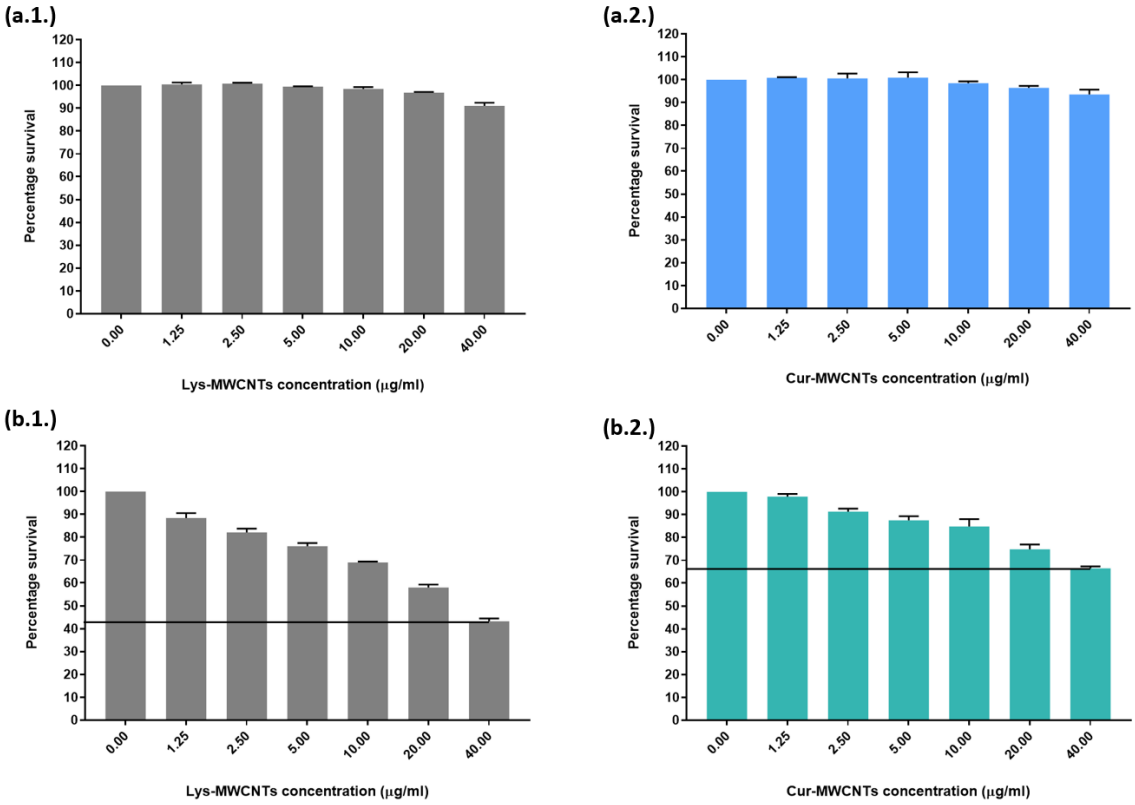
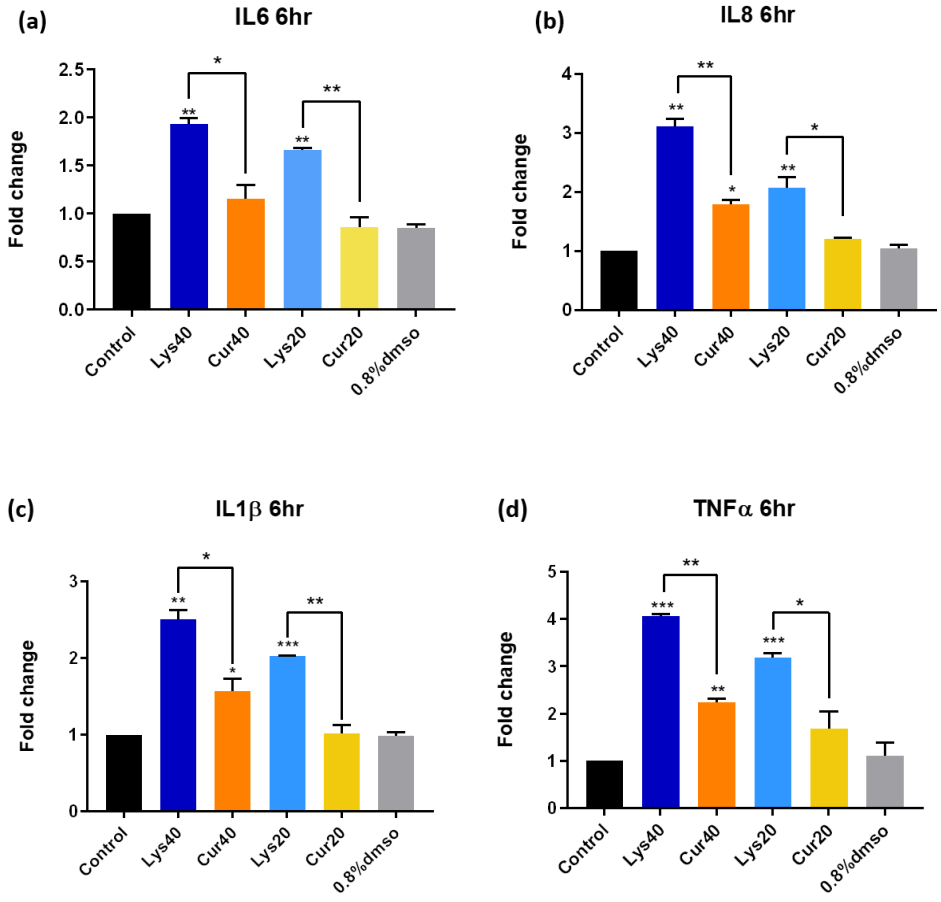


Figure 6. Cell survival percentage graph

(a) after 24 hours incubation with (a.1.) Lys-MWCNTs and (a.2.) Cur-MWCNTs and (b)) after 48 hours incubation with (b.1.) Lys-MWCNTs and (b.2.) Cur-MWCNTs.

4.2. Curcumin coating on the surface of MWCNTs reduces its Inflammatory effects

In order to check if Curcumin coating on the surface of MWCNTs is effective in reducing inflammation, we performed qRT-PCR and Western blot to check the relative change in gene expression and protein expression. In this study, we incubated the cells with two concentrations of Curcumin-coated and uncoated MWCNTs- 40 µg/ml and 20 µg/ml. A 0.8% DMSO control was also kept so as to confirm that any of the changes seen were not a result of its effect. The incubation time selected for this study was 6 hours as previous literature has reported peak expression of inflammatory markers at this time point.



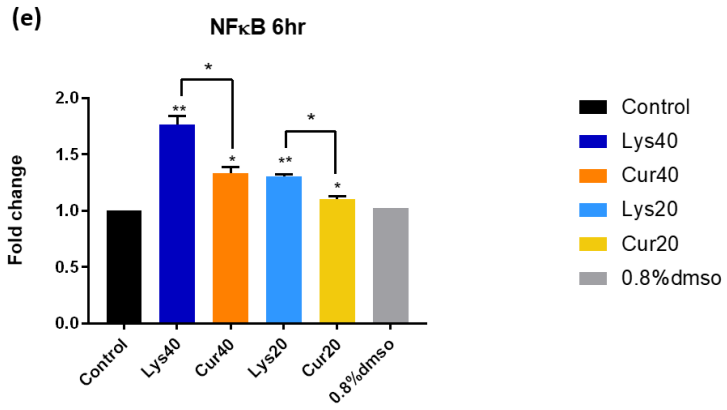


Figure 7. Relative change in gene expression of inflammatory markers after 6 hours of incubation (a) IL-6, (b) IL-8, (c) IL-1β, (d) TNFα and (e)NFκB

4.2.1. Curcumin coating reduced the expression of Inflammatory genes after 6 hours of exposure to MWCNTs

We selected a panel of 5 inflammatory markers which play a major role in inflammation caused via MWCNTs exposure as known by literature survey[40]. In uncoated samples, 6hr exposure to MWCNTs at 40 µg/ml increased the expression of IL-6 by ~2 folds while in Curcumin-coated MWCNTs, at the same concentration IL-6 expression was increased to only ~1.2 folds **(Fig. 7. a)**. A similar decrease could be observed at 20 µg/ml concentration as well. Similarly, the expression of the other two cytokines- IL-8 **(Fig. 7. b)** and IL-1β **(Fig. 7. c)** also decreased in a familiar pattern at both 40 µg/ml and 20 µg/ml concentrations. NFκB, **(Fig. 7. e)** which is the key molecule regulating the inflammatory cascade, showed a similar pattern. At 40 µg/ml its relative expression in uncoated samples was 1.77 folds while in Curcumin-coated ones it dropped to 1.3 folds. TNFα gene expression **(Fig. 7. d)** dropped from 4 to 2.2 folds. 0.8% DMSO control showed non-significant change in gene expression for all the 5 genes.

4.2.2. Curcumin coating reduced the expression of Inflammatory proteins after 6 hours of exposure to MWCNTs

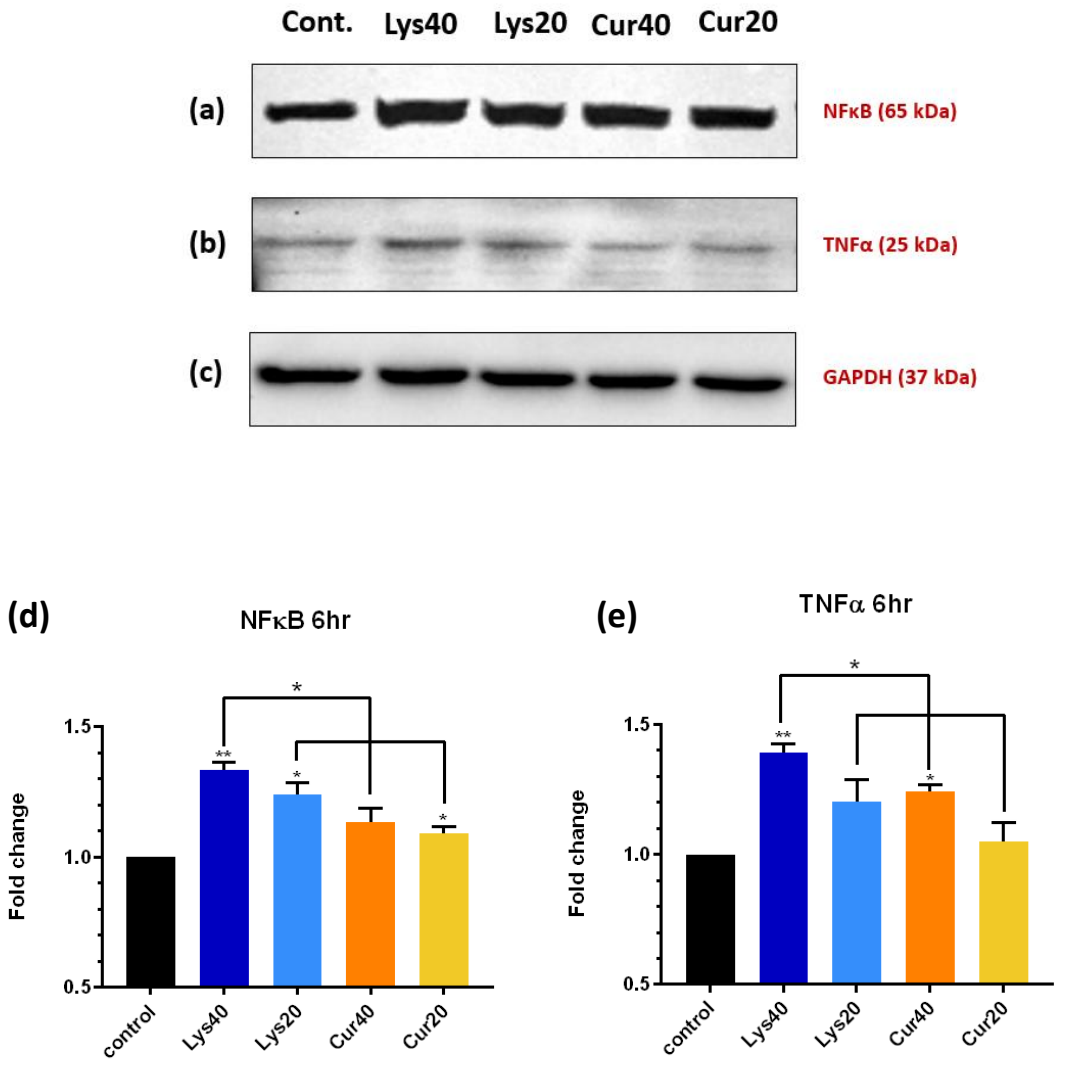


Figure 8. Relative change in expression of inflammatory proteins after 6 hours exposure to MWCNTs (a) NFκB blot, (b) TNFα blot (c) GAPDH blot, (d) fold change in NFκB and (e) fold change in TNFα

Fold change in protein expression of inflammatory markers has been normalised with GAPDH. In uncoated samples, 6-hour exposure to MWCNTs at 40 μg/ml increased the expression of NFκB by ~1.3 folds while in Curcumin-coated MWCNTs, at the same concentration NFκB expression was increased to only ~1.1 folds **(Fig. 8. d)**. 0.8% DMSO control showed non-significant fold change in expression of NFκB with respect to control.

Similarly, in TNFα **(Fig. 8. e)** in uncoated samples, 6-hour exposure to MWCNTs at 40 μg/ml increased the expression by ~1.4 folds while in Curcumin-coated MWCNTs, at the same concentration TNFα expression was increased to only ~1.2 folds. 0.8% DMSO control showed non-significant fold change in expression of TNFα with respect to control.

4.3. Curcumin coating on the surface of MWCNTs reduces Oxidative stress caused due to their exposure

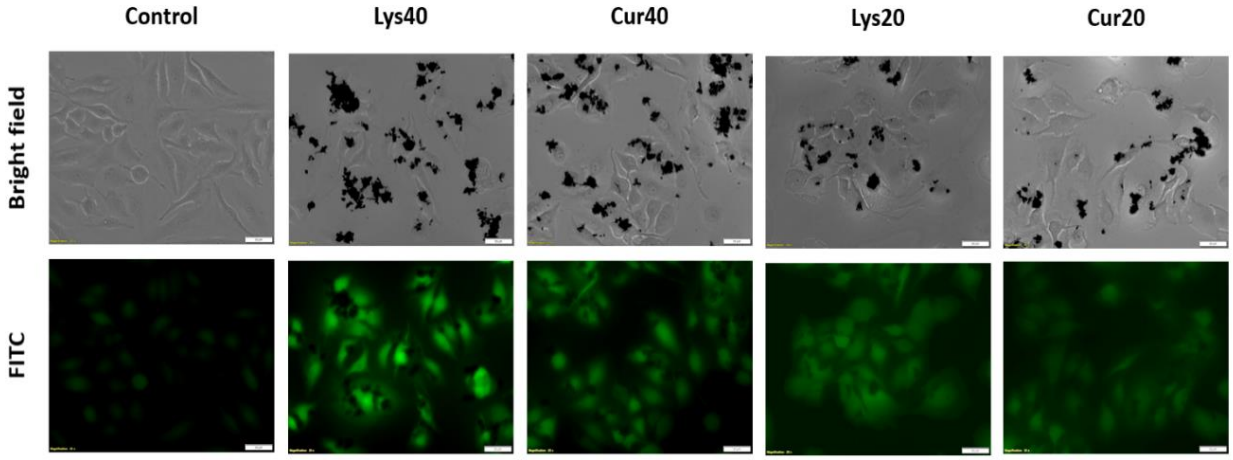
In order to check if coating of Curcumin, which is a well-known antioxidant compound, is able to reduce the oxidative stress on the cells by reducing ROS (Reactive Oxygen Species) production, we performed DCFDA staining and Western blot of antioxidant enzyme Catalase.

4.3.1. Curcumin coating successfully reduced cellular ROS production in A549 cells after 12 hours of incubation with MWCNTs

From the images of DCFDA staining **(Fig. 9. a)** it could be clearly seen that the fluorescence intensity was highest in case of Lys40 sample i.e., 40 μg/ml of uncoated MWCNTs. It was measured to be 14.2 folds higher as compared to control **(Fig. 9. b)**. While in Curcumin-coated MWCNTs at the same concentration, fold change in ROS was as low as 9.6 folds. Similarly, at lower concentration of 20 μg/ml, Curcumin-coated MWCNTs showed lesser ROS (8.7 folds) than the uncoated ones (5.6 folds).

This proves that coating of Curcumin was able to significantly lower cellular ROS production at both 40 μg/ml and 20 μg/ml. Also, it is evident that there is a concentration dependent decrease in cellular ROS production in both Curcumin coated and uncoated MWCNTs.

(a)



(b)

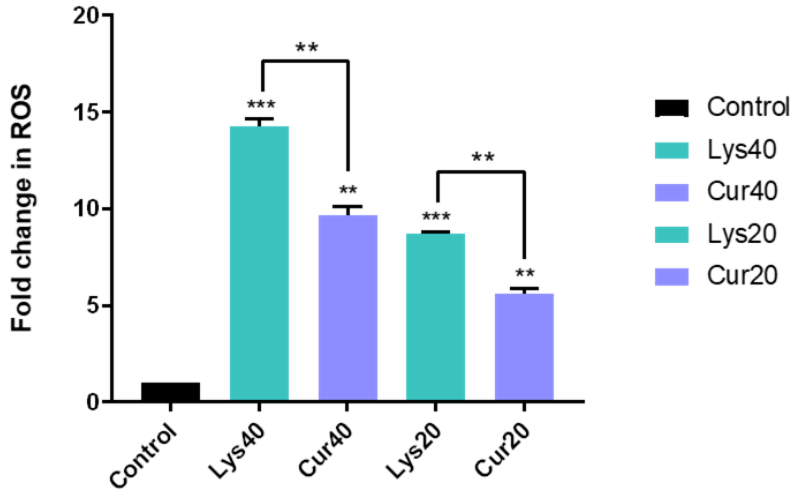


Figure 9. Cellular ROS estimation by DCFDA staining (a) reference images after 12 hours of exposure to MWCNTs (b) Fold change in cellular ROS produced

4.3.2. Curcumin coating helped to attenuate the lowering of Catalase expression in A549 cells post 12 hours of exposure to MWCNTs

Catalase is an antioxidant enzyme found in the peroxisomes of mammalian cells. The function of catalase is to breakdown Hydrogen peroxide into water and oxygen. It is a key enzyme involved in regulation of cellular ROS balance. In our study after 12 hours of incubation of A549 cells with MWCNTs it was found that the expression of catalase enzyme had decreased to nearly 0.5 folds in Lys40 sample **(Fig. 10 c).** Curcumin here showed a rescue effect and brought up the catalase expression to 0.85 folds, which is a significant increase in its expression. A similar effect was observed in the 20 μg/ml concentration of both groups. The fold changes were measured with respect to GAPDH which is constitutively expressed in all cells. The decrease in expression of Catalase enzyme can be correlated to the increase in ROS activity in the uncoated MWCNT treated cells.

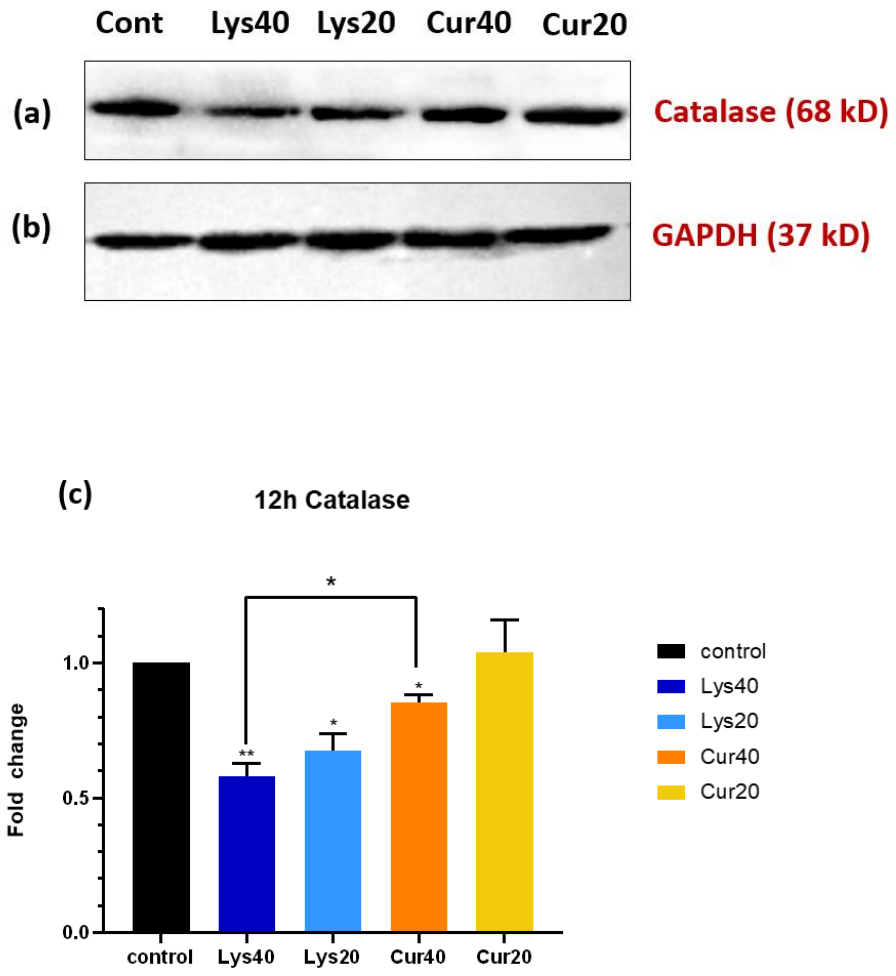


Figure 10. Western blot representative images

(a) Catalase and (b) GAPDH.

(c) Relative change in protein expression of Catalase after 12 hours of exposure to MWCNTs

4.4. Curcumin coating prevented the loss of Mitochondrial membrane potential in A549 cells caused due to exposure of MWCNTs for 12 hours.

Mitotracker green-red staining is a technique used to evaluate the changes in mitochondrial membrane potential (MMP). Mito-green stains all mitochondria equally hence it indicates mitochondrial mass or number of mitochondria in cell. Mito-red stains mitochondria based on its membrane potential. Higher potential leads to more binding of the dye while lower potential leads to less binding. In our experiment it was observed **(Fig. 11. b)** that all three groups- Control, Lys40 and Cur40 had nearly constant green fluorescent signal intensity indicating that there was no change in number of mitochondria in cell after MWCNTs exposure. However, it could be observed **(Fig. 11. c)** that Lys40 had significantly lowered Mito-red fluorescent intensity. This indicates that the mitochondria in Lys40 group had lesser mitochondrial membrane potential. Low MMP indicates damaged or malfunctional mitochondria. So, we can infer that exposure to MWCNTs for 12 hours caused damage to mitochondria which can be the reason for higher ROS production.

On the other hand, we can observe that in Cur40 group the Mito-red fluorescent intensity was significantly higher than that of Lys40 group. This indicates that Curcumin coating is able to prevent mitochondrial damage and hence is able to prevent ROS imbalance in the cells.

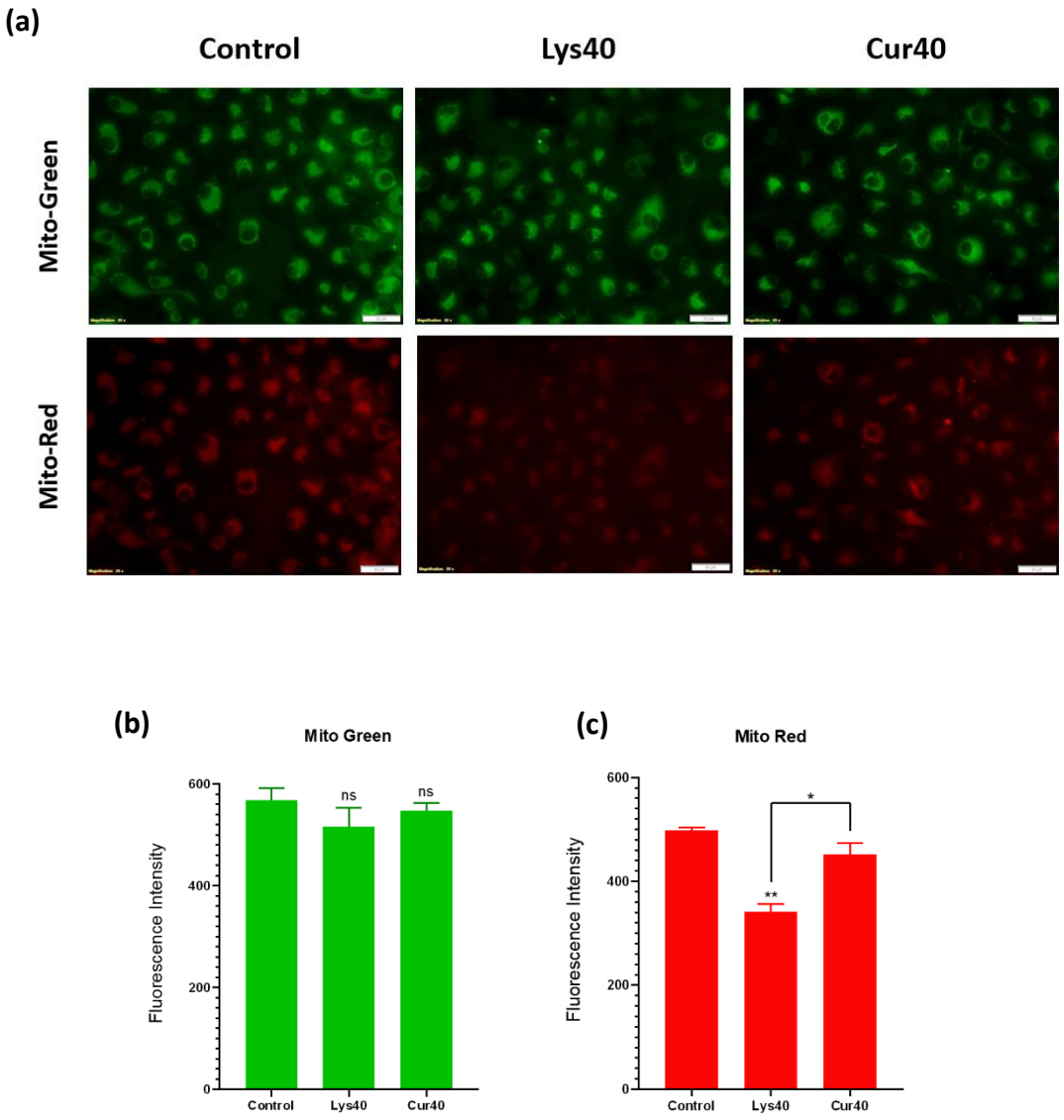


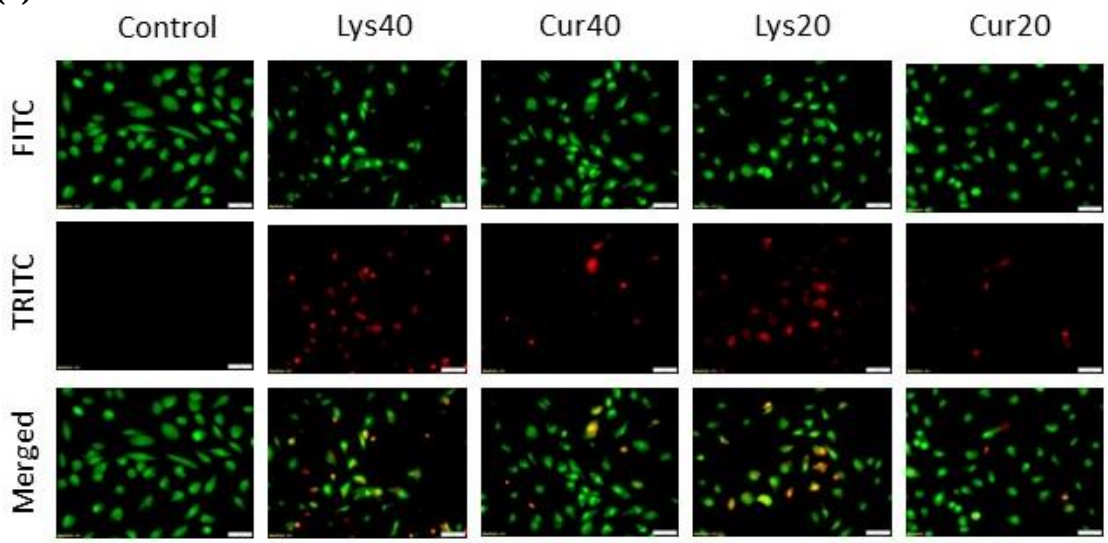
Figure 11. (a) Mitotracker Green-Red staining representative images after 12 hours of incubation with MWCNTs (b) Fluorescence intensity of Mito-green (c) Fluorescence intensity of Mito-red

4.5. Curcumin coating on the surface of MWCNTs reduces Apoptosis caused due to their exposure

Ethidium Bromide (EB) and Acridine Orange (AO) dual fluorescent staining method was performed to check if Curcumin coating was able to delay Apoptosis in A549 cells after exposure for 48 hours.

It could be observed **(Fig.12.b)** that the percentage of live cells was significantly lower in the Lys40 (32%) and Lys20 (47%) sample groups in comparison to Cur40 (60%) and Cur20 (65%) samples. The difference in the number of Early Apoptotic cells (EACs) was non-significant in all the groups. The percentage of Late Apoptotic cells (LACs) was higher in uncoated MWCNT groups and lower in Curcumin coated groups, however, the decrease was significant only in the 20 μg/ml set. The percentage of dead cells was highest in Lys40 group (22.5%) and it was significantly lowered in Cur40 group (7.4%). The results of EB/AO staining revealed that Curcumin coating was indeed effective in delaying apoptosis in A549 cells. There might be several reasons for this effect like the cytoprotective effect of Curcumin on the cells, the fact that ROS activity was reduced in the Curcumin-coated samples. Inflammation was also lesser in the Curcumin-coated samples. All these factors contribute to an overall reduction in cell death as oxidative stress and inflammation are two major events which lead to apoptosis.

(a)



(b)

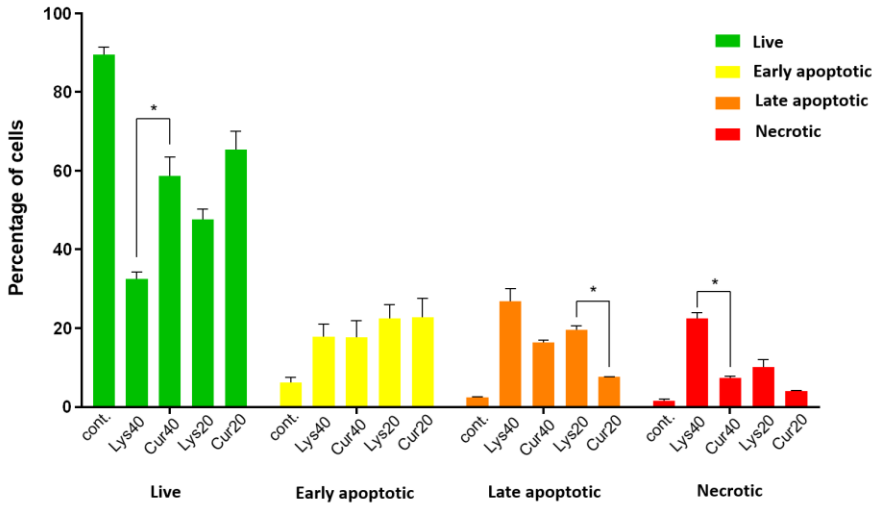


Figure 12. (a) EB/AO fluorescent staining images 48 hours post exposure to MWCNTs. (b) Distribution of cells amongst different cell stages – Live, early apoptotic, late apoptotic and necrotic.

Chapter 5: CONCLUSION AND SCOPE FOR FUTURE WORK

5.1. CONCLUSION

Carbon nanotubes are promising agents for tissue engineering, in-vivo imaging techniques and targeted drug delivery. However, they need certain physical or chemical modifications to eliminate their negative effects on the living systems like inflammation, oxidative stress and apoptosis. In our study, we have proved that coating of Curcumin, which is a natural medicinal plant compound, can help in mitigating the harmful effects of these MWCNTs. When cells are exposed to Curcumin coated MWCNTs, the Curcumin molecules are slowly released into the cells. Curcumin coating on MWCNTs was successful in lowering the expression of inflammatory markers like IL-6, IL-8, IL-1β, TNFα and NFκB at both transcript and protein level. This was probably due to inhibition of NFκB by Curcumin. DCFDA staining revealed that Curcumin coating lowered down cellular ROS production by increasing the expression of Catalase, a major antioxidant enzyme. It also showed a recovery effect and helped in maintaining the mitochondrial membrane potential. This might contribute in maintaining the cellular ROS balance. This leads to lowering of oxidative stress in the cells. Reduced inflammation and reduced oxidative stress together led to the eventual reduction in cell death in the cells as compared to its uncoated counterparts. **Figure 13** shows a diagrammatic representation of the effect of Curcumin coated MWCNTs on cells.

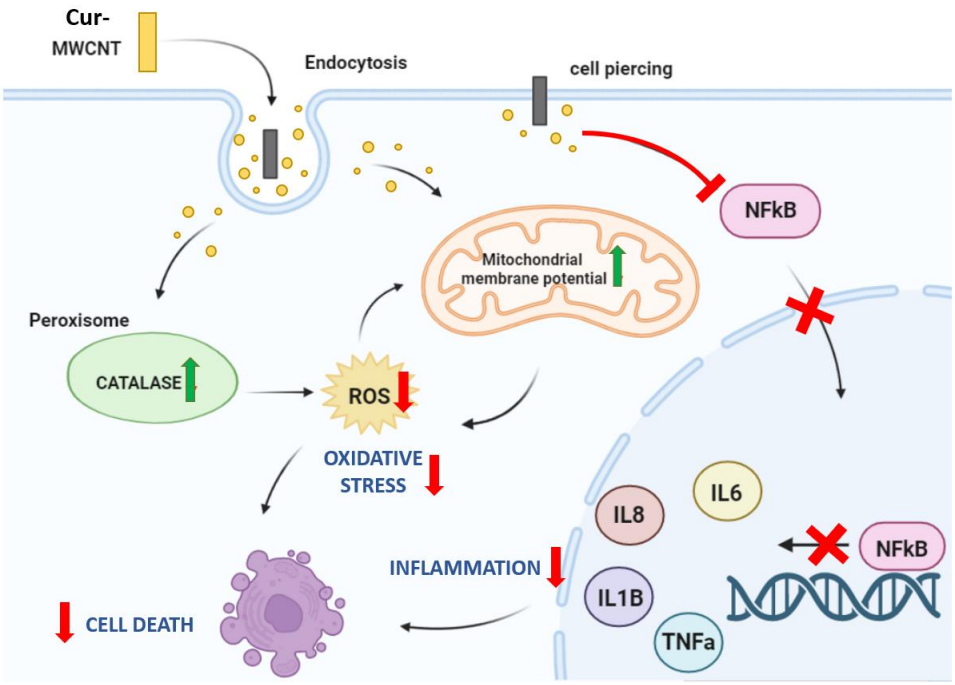


Figure 13. Effect of Curcumin coated MWCNTs on cell.

5.2. SCOPE FOR FUTURE WORK

Surface coating technology is an emerging field of nanotechnology. Scientists around the globe are working towards finding biocompatible compounds which can be coated on the surface of nanomaterial like nanoparticles and nanotubes in order to improve their properties and efficiencies. Using plant-based compounds is a smart choice for such coatings[41]. The reason being that they are largely biocompatible and come with a wide variety of beneficial properties. In future, more such suitable compounds can be screened for their desired properties and checked for their compatibility with the nano-systems. Many studies have reported that surface coatings at times add certain downgrading properties to the nanomaterials, so one should be careful while screening and testing of such compounds. Also, there are limitations to bio-based surface coatings on nanotubes like detachment of coated compound upon dissolution in

organic solvents and low stability on pH and temperature fluctuations[42]. Our collaborators have conducted a 120 hours stability study for Curcumin coated MWCNTs in which they found that the Curcumin coating detachment percentage was around 15-20% after 120 hours of incubation in phosphate buffered saline (PBS) at pH 7.4 and 37°C. This is a good attachment stability for Curcumin though it can definitely be improved. The structure of Curcumin can be chemically modified such that it retains its anti-inflammatory, antioxidant and other properties and at the same time increases its attachment efficiency and stability over wide range of pH and temperatures. In future there is scope for improving the coating efficiency so that more compound can be coated per gram of CNTs. Compounds with greater biocompatibility and useful properties can be screened and used. And lastly, the stability of such attachments or coatings can be improved such that they remain functional over a wide range of pH, temperatures and for a longer duration of time.

ANNEXURE-A

LIST OF PRIMERS

Sr. No.	Gene	Forward and Reverse primer
1	GAPDH	FP: TGCACCACCAACTGCTTAG RP: GATGCAGGGATGATGTTC
2	IL-6	FP: TACCCCCAGGAGAAGATTCC RP: TTTTCTGCCAGTGCCTCTTT
3	IL-8	FP: GTGCAGTTTTGCCAAGGAGT RP: CTCTGCACCCAGTTTTCCTT
4	IL-1β	FP: GGGCCTCAAGGAAAAGAATC RP: TTCTGCTTGAGAGGTGCTGA
5	NFκB	FP: GAACAGCCTTGCATCTAGCC RP: TCCGAGTCGCTATCAGAGGT
6	TNFα	FP: CAGAGGGCCTGTACCTCATC RP: GGAAGACCCCTCCCAGATAG

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