INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com ISSN 2230 – 8407
Research Article

CYTOTOXICITY INVESTIGATION ON CULTURED CT3 CELLS TREATED WITH MWCNTS AND F-MWCNTS

A.Malarvizhi[1] and A.Pandurangan[2]*
[1]Department of Chemistry, Anna University, Guindy, Chennai-600 025, India
[2]Institute of Catalysis and Petroleum Technology, A. C. Tech, Anna University, Chennai-600 025, India

Article Received on: 12/12/11 Revised on: 31/01/12 Approved for publication: 11/02/12

*Email: pandurangan_a@yahoo.com

ABSTRACT
Functionalised carbon nanotubes (*f*-MWCNTs), derived from Multiwalled carbon nanotubes (MWCNTs) was synthesized and examined their cytotoxicity effect in drug release applications. MWCNTs were synthesized by chemical vapor deposition over Fe -MCM-41 molecular sieves using acetylene as a carbon source. They were characterized by XRD, FT-IR, RAMAN, SEM, TEM and functionalized using H_2SO_4:HNO_3 mixtures. The MWCNTs and f-MWCNTs were tested for "invitro" MTT assay on cultured CT3 cells at different concentrations (0, 5, 10, 15 and 20 mg/ml) for 12-72 hrs. The results confirmed that *f*-MWCNTs were less toxic than MWCNTs. This observation suggests the applicability of *f*-MWCNTs in drug release applications. For the treatment of Hyperlipidemia and cholesterol control, f-MWCNTs could be novel potential materials.
Keywords: Carbon nanotubes, Functionalised CNT, Toxicity –MTT Assay

INTRODUCTION

The emerging field of nanobiotechnology bridges physical sciences with biological sciences via chemical methods, in developing novel tools and platforms for understanding biological systems and disease diagnosis and treatment [1-3]. Carbon nanotubes (CNTs) are one of the major building blocks of this new technology. Because of their excellent physical properties (high tensile strength, ultra-light weight, thermal and chemical stability, metallic and semi-conductive electronic properties), CNTs have sparked a great research interest and are being developed for multiple commercial applications including biosensors, molecular transporters for drug delivery and novel biomaterials[4]. The application of functionalized carbon nanotubes as new nanovectors for drug delivery was apparent immediately after the first demonstration of its ability to penetrate into cells. Carbon nanotubes can be used to deliver their cargoes to cells and organs [5, 6]. However, the interaction between cells and carbon nanotubes (CNT) is a critical issue that will determine any future biological application of such a structure [7].

Owing to the lack of solubility and dispersion of CNTs in an aqueous solution, unmodified CNTs disperse poorly in water but easily float in air. Recent, fundamental studies or applications, reported that surfactants are often used to make CNTs disperse homogeneously in a medium, which are tunable and these surfactants do not influence the toxicological behavior of the CNTs[8]. This suggests that the surface behaviour/characteristics of CNTs play an important role in deciding the dispersion, solubilization, biocompatibility enhancement, and reduction of the toxicity of CNTs. Hence the biomedical functions of CNTs are largely dependent on surface chemistry. More interestingly, biological behaviors including toxicity and biomedical functions of CNTs are largely dependent on the process of their synthesis, purification, and functionalization. Recent reviews has well summarized that the covalent chemistry is required for surface modification of CNTs [9].

The sidewall surface of pristine CNTs is highly hydrophobic, and bundles are formed mainly because of van der Waals interactions between the sidewalls of the individual tubes [10]. Surface functionality of CNTs [11] and individually dispersed CNTs in biocompatible media are very important for biological applications. Proteins and enzymes are absorbed by carbon nanotubes [12, 13].

Oxidation is carried out with strong oxidizing agents such as concentrated HNO_3, H_2SO_4 [14, 15]. After surface modification, the hydrophilic CNTs generally become less toxic to human T cells than the unmodified CNTs. However the toxicity of pristine hydrophobic CNTs was lower than that of oxidized MWCNTs, most likely due to their better dispersion in aqueous solution. At higher dose (400 mg ml^{-1}), the oxidized MWCNTs become more toxic and induced a reduction in cell viability [16]. As a new strategy, oxidized CNTs also been functionalized by 1, 3-dipolar cycloaddition for the double functionalization of CNTs to assess the characteristics of toxicity and uptake of CNTs functionalized with the antibiotic Amphotericin B (AmB) and fluorescein towards mammalian cells, and to evaluate the antifungal activity of CNT–AmB conjugates [17].

Safety is the first requirement of any material to be used in medicine. On the other hand, raw carbon nanotubes were shown to be toxic to mice after inhalation into the lung [18, 19]. As grown, raw carbon nanotubes have highly hydrophobic surfaces, and are not soluble in aqueous solutions. Recent research showed that unfunctionalized, long MWCNTs may have a carcinogenic threat in mice [20]. It appears that raw CNTs and f- CNTs without serum-stable functionalization show toxicity to cells at a moderate dosage, while serum-stable, functionalized CNTs show little toxicity even at high dosages.

The present study investigated the efficacy of CNT-based nanostructures on cell viability and toxicity. The CNTs synthesized by CVD method, were purified and functionalized by introducing COOH groups in it .The functionalized MWCNTs were characterized by XRD, FT-IR, Raman spectroscopy, SEM and TEM. The surface modified MWCNTs were investigated for cytotoxicity and biocompatibility against CT3 cells by MTT assay method and compared to non functionalized MWCNTs.

MATERIALS AND METHODS

Materials

Purified multiwall carbon nanotubes were produced by Chemical vapor deposition method. H_2SO_4 and HNO_3 were purchased from Merck chemicals. Human CT3 cell lines

were purchased from life bio-tech Institute, Chennai, India. They are supplemented with 10% fetal calf serum (FCS, Gibco BRL), penicillin (100U/ml), streptomycin (100µg/ml), L-glutamine (2mM) (ICN Biomedicals, Costa Mesa, CA, USA) and amphotericin B 2.5 µg/ml (Sigma-Aldrich). Culture medium contains 10% fetal bovine serum with RPMI1640 culture medium, the CT3 cells were cultured under the conditions of 37 °C, 5% CO_2.

Synthesis Of Carbon Nanotubes

Synthesis of CNTs was carried out in a horizontal furnace under atmospheric pressure. About 100 mg of the catalyst was spread on a long quartz boat placed inside a quartz tube. The reaction mixture containing acetylene and nitrogen gas was passed over the catalyst bed for a predetermined time. The experiments were carried out from 650° to 850°c. The percentage of carbon deposited by the catalytic decomposition of acetylene was obtained from the following equation:

$$\text{Carbon deposit (\%)} = 100 \times (m_{tot} - m_{cat})/m_{cat}$$

Where, m_{cat} and m_{tot} are the mass of the catalyst before and after reaction respectively.

Purification Of Carbon Nanotubes

The synthesized material was treated with 40 % hydrofluoric acid at ambient temperature in order to remove silica phase. The obtained samples were immersed in nitric acid to remove metal catalysts and amorphous carbon, because nitric acid (HNO_3) is inexpensive and fairly effective in removing metal catalysts and amorphous carbon from large quantities of raw material. Many Researchers [21, 22] have reported the purification procedure by acid treatment. As the metal particles can accumulate in cell causes damage, this process is essential to remove these particles.

Preparation Of Functionalised MWCNTS

Oxidised MWCNTs (MWCNTs-COOH) were obtained by refluxing MWCNTs in concentrated HNO_3 for 28hrs. The as-prepared MWCNTs-COOH (100mg) was suspended in 15ml of DMF solution, sonicated for 50 min, and purified by washing with distilled water. The resulting functionalized CNTs (f-CNTs) were dried overnight in vacuum between 80 and 90 °C.

***In Vitro* Cytotoxicity**

The cytotoxicity of MWCNT and MWCNT-COOH, were assessed by standard MTT assay. The CT3 cells at a concentration of one lakh/ml/well (10 cell/ml/well) were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO_2. Cells in the exponential growth phase were seeded in 96-well plates (Nunc, Denmark) at a density of 1×10^4 viable cells/well. After overnight incubation, 0, 5, 10, 15 and 20µg/ml MWCNT and MWCNT-COOH in dimethyl sulfoxide at 37°C were introduced into cell cultures for predetermined times under standard cell culture conditions. At designated time intervals, 5µL of MTT (10 mg/mL) was added to each well and incubated for 12–72hrs. The formazan crystals in each well were dissolved in 100µL of dimethyl sulfoxide. The absorbance of each well was measured with a microplate reader (Anthos 2020, [Anthos Labtec Instruments GmbH, Salzburg, Austria]) at 570 nm. The percentage of cell viability was calculated using the following equation:

$$\text{Cell viability} = \frac{\text{Abs 570nm of treated group}}{\text{Abs 570nm of control group}} \times 100$$

RESULTS AND DISCUSSION

XRD

The XRD pattern of the as-synthesized and acid purified carbon nanotubes are shown Fig.1a. It shows a broad diffraction peak at 26° with additional peaks at 32°, 44.50° and 54.14° correspond to reflections of graphite Fe_2O_3 phases. Fig. 1b shows the XRD pattern of acid purified sample, indicating the removal of Fe_2O_3 phases completely after acid treatment.

FT-IR

The presence of carboxylic group (-COOH), carbonyl group (C=O), and hydroxyl group (O-H) were confirmed by FT-IR spectra. The oxidation of the Multiwalled carbon nanotubes were confirmed by the presence of carboxylic groups in them[23]. Fig 2a, b shows the spectra of MWCNTs before and after oxidation with concentrated acid. The oxidized MWCNTs given in Fig 2b, indicates the presence of a sharp peak at 1710 cm^{-1}, due to C=O stretching of COOH group and a broad band at 3380 cm^{-1} due to OH group.

SEM IMAGE

The SEM images of purified CNTs are shown in Fig 3 a, b. In Fig 3a large number of CNTs long with traces of metal particles and amorphous carbon were found in the grown samples. Fig 3 b shows the ruptured nanotubes developed in the sidewall defects.

TEM IMAGE

Transmission electron microscope was used to investigate the structure and morphology of MWCNTs before and after chemical modification. The TEM image of MWCNTs given in Fig. 4a showed the presence of metal particles and impurities with an average outer diameter of 20 – 30 nm. The TEM image of MWCNTs after acid treatment is given in Fig. 4b. The surface of the nanotubes is clean indicating the removal of impurities and metal particles after the treatment

MTT VIABILITY

Initial images of the Vero cells and particles without stain imaged using visible light are depicted in Fig. 5a. It shows that the particles were interacting with the cells, and there was a change in cellular morphology when compared to the control. The cytotoxicity of MWCNTs before and after functionalisation was assessed by an MTT assay of cell lines (CT3). After incubation at various concentrations (0, 5, 10, 15 and 20mg/ml) of both MWCNTs and MWCNTs-COOH for different durations (12, 24, 48 and 72 hrs) with cells, an MTT assay was performed to evaluate the viability of CT3 cells and the result was given in fig 5(a & b). The data was taken for 12, 24, 48 and 72hrs time points. The cell viability data was given in Table 1. According to the 72 hrs time point data, only 26 % cell viability is observed in the presence of MWCNTs, whereas the majority of cells remained alive upon treatment with MWCNTs-COOH. As can be seen in Fig. 5 (b) the percentage of cell viability is more than 72% when cells were treated with MWCNTs-COOH showing significant increase in cell viability compared to non-functionalized MWCNTs. However, an expected dose-response relationship is observed. At higher concentration of MWCNTs, regardless of 12, 24, 48 and 72hrs point's data, continuous reinforcing effect on the cells were observed. The initial invitro studies indicate that, functionalization of MWCNTs reduces toxicity and improves biocompatibility of cells. The excellent cell viability in the presence of f-MWCNTs indicates that they can be employed in drug release applications and in the treatment of Hyperlipidemia and cholesterol control. The performance of *f*-MWCNT in drug release and in the treatment of Hyperlipidemia was under investigation and is

showing promising results. The results of the study will be reported after complete investigation.

CONCLUSION

Pure MWCNTs and functionalized MWCNTs were prepared and their activity was studied in CT3 cells and compared against Vero cells. FT-IR spectroscopy confirmed the incorporation of COOH groups on MWCNTs. The cytotoxicity studies on CT3 and Vero cells revealed that *f*-MWCNT is less toxic than MWCNT and the cell viability was concentration dependant. Due to less cytotoxicity, the f-MWCNTs can be concluded as biocompatible and can be used for further cell-line study.

ACKNOWLEDGMENT

One of the authors, Mrs. A. Malarvizhi is thankful to UGC, New Delhi, for the award of Rajiv Gandhi Fellowship.

REFERENCES

1. Whitesides GM. The right size in nanobiotechnology.Na. Biotech. 2003; 21: 1161 -65.
2. Lowe CR. Nanobiotechnology: The fabrication and applications of chemical and biological nanostructures.Curr Opin Chem Biol. 2000; *10:* 428- 434.
3. Wang L, Zhao W, Tan W. Bioconjugated silica nanoparticles: Development and applications. Nano Res. 2008; 1: 99 -115.
4. Lin Y, Taylor S, Li H, Fernando SKA, Qu L, Wang W et al.,. Advances towards bioapplications of carbon nanotubes. J Mater Chem. 2004; 14: 527-541.
5. Gannon GI. Carbon nanotubes-enhanced thermal destruction of cancer cells in a noninvasive radiofrequency field. Cancer. 2007; 110:2654–2655.
6. Liu Z, Sun X, Nakayama-Ratchford N, Dai H. The supramolecular chemistry of organic- inorganic hybrid materials. ACS Nano. 2007, 1:50–56.
7. Kostarelos k, Lacerda L, pastorin G, Wu w, Wieckocoski S, Luangsivilay J et al.,. Cellular up take of functionalised Carbon nanotubes is independent of functional group and cell type. Nature nanotech. 2007; 2:108-113.
8. Cherukuri P, Gannon C J, Leeuw T K, Schmidt H K, Smalley R E,Curley S A et al.,. Mammalian pharmacokinetics of carbon nanotubes using intrinsic near- infrared fluorescence. Proc Natl Acad Sci U. S. A. 2006; 103: 1888- 2.
9. Wu H C, Chang X L, Liu L, Zhao F and Zhao Y L. Chemistry of carbon nanotubes in biomedical applications .J Mater Chem. 2010; 20: 1.
10. Tasis D, Tagmatarchis N, Bianco A, Prato M. Chemistry of carbon nanotubes. Chem Rev.2006; 106:1105–1136.
11. Munge B , Liu GD, Collins G, Wang J. Multiple enzyme layers on carbon nanotubes for electrochemical detection down to 80 DNA copies. Anal Chem. 2005; 77:4662–4666.
12. Zhang B , Xing Y, Li Z, Zhou H, Mu Q, Yan B. Functionalized carbon nanotubes specifically bind to a-chymotrypsin's catalytic site and regulate its enzymatic function. Nano Lett. 2009; 9: 2280–2286.
13. Gao Y, Kyratzis I. Covalent immobilization of proteins on carbon nanotubes using the cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide—a critical assessment. Bioconjug Chem. 2008; 19: 1945–1950.
14. Niyogi S, Hamon M A, Hu H, Zhao B, Bhowmik P, Sen R et al.,. Chemistry of Single-Walled Carbon Nanotubes . Acc Chem Res. 2002; 35: 1105.
15. Rosca I D, Watari F, Uo M, Akaska T. Oxidation of multiwalled carbon nanotubes by nitric acid. Carbon. 2005; *43*: 3124- 3131.
16. Bottini M, Bruckner S, Nika K, Bottini N, Bellucci S, Magrini A, Bergamaschi A and Mustelin T. Multi-walled carbon nanotubes induce T lymphocyte apoptosis Toxico let. 2006; 160: 121.
17. Wu W, Wieckowski S, Pastorin G, Benincasa M, Klumpp C, Briand J, Gennaro R, Prato M, Bianco A .Targeted Delivery of Amphotericin B to Cells by Using Functionalized Carbon Nanotubes .Angew Chem. 2005; Int. *Ed* 44:6358.
18. Lam C W, James JT, McCluskey R, Hunter R L. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after in tratracheal instillation. Toxicol Lett. 2004; *77*: 126- 134.
19. Warheit DB, Laurence BR, Reed KL, Roach DH, Reynolds GAM, Webb TR. Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. Toxicol Lett. 2004; 77: 117 -125.
20. Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WAH, Seaton A et al.,. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat. Nanotech. 2008; *3:* 423- 428.
21. Chiang W, Brinson BE, Smalley RE, Margrave JL, Hauge RH. Purification and Characterization of Single-Wall Carbon Nanotubes (SWNTs) Obtained from the Gas-Phase Decomposition of CO (HiPco Process) J Phys Chem B. 2001; 105: 1157.
22. Park TJ, Banerjee S, Benny TH, Wong SS. Using Microscopy as a Visualization Tool for Purity Evaluation in Single-Walled Carbon Nanotubes (cover) J Mater Chem. 2006; 16: 141.
23. Foldvari, Bagonluri M. Carbon nanotubes functional excipients for nanomedicines: I.pharmaceutical properties. Nanomed Nanotech Bio& Med. 2008; 4: 173-182 .

Table 1: **MTT analysis of cell viability**

sample	12hrs	24 hrs	48 hrs	72 hrs	Viability%
Control	100	100	100	100	100
MWCNTs	48	38	3	26	26
MWCNTs-COOH	86.88	80	75	72	72

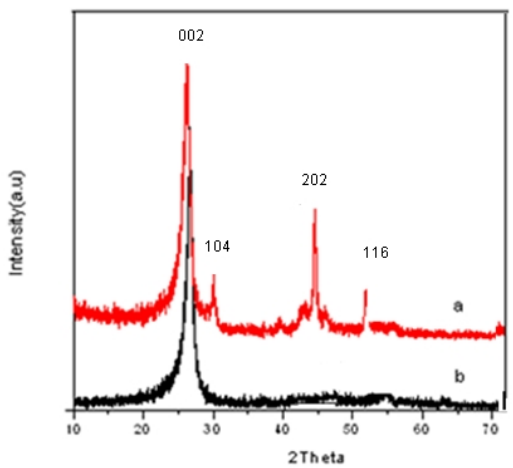

Fig.1. XRD patterns of the carbon nanotubes samples (a) as-synthesized (b) after purification

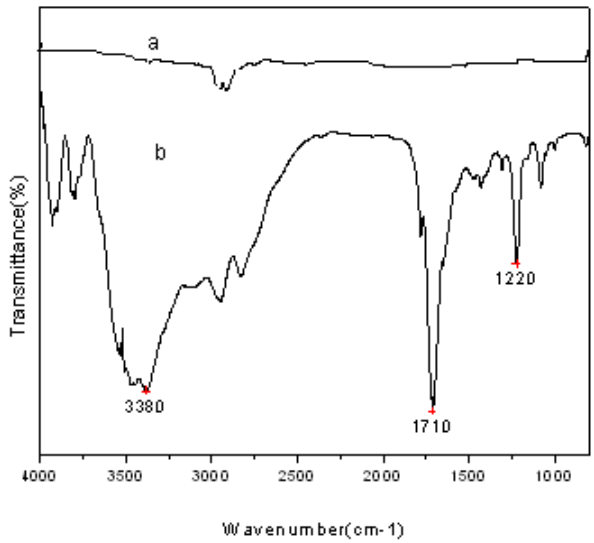

Figure 2 Fourier transform spectra (a) raw MWCNTs and (b) MWCNTs-COOH

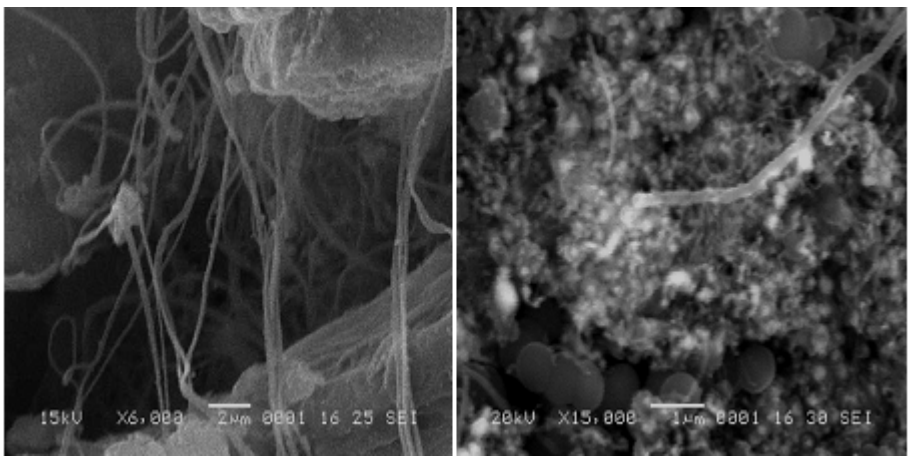

Figure 3 SEM Image of (a) MWCNTs and (b) *f*-MWCNTs

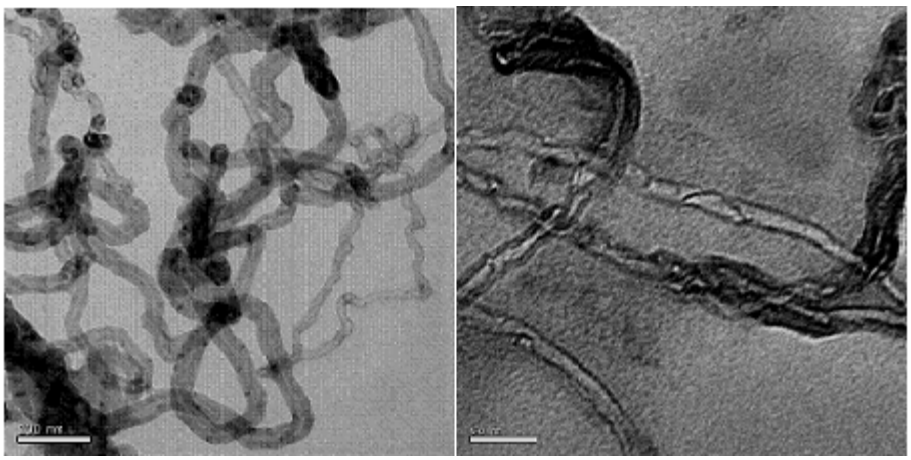

Figure 4 Shows the TEM image of (a) MWCNTs and (b) f-MWCNTs

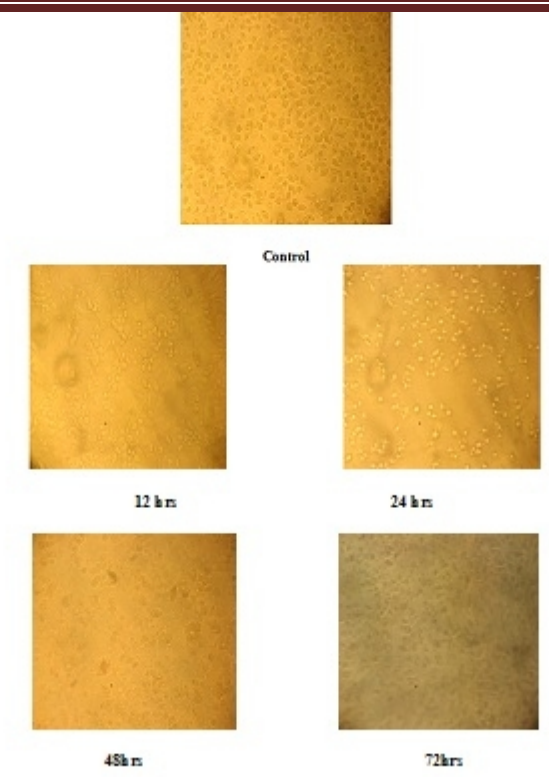

Figure 5 (i).Confocal images of CT3 cells before functionalised in solution of MWCNTs control (a) 12 hrs (b) 24 hrs (c) 48hrs (d) 72 hrs.

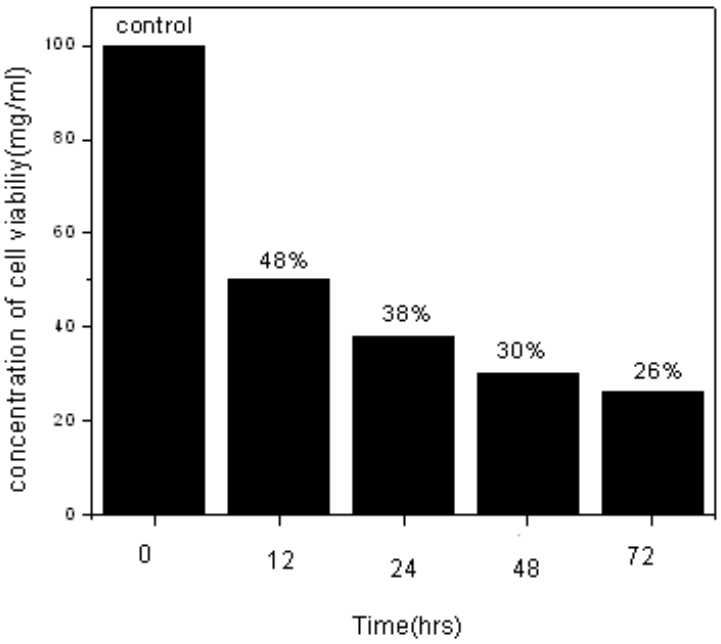

Figure 5 (i) MTT analysis of cell viability (MWCNTs)

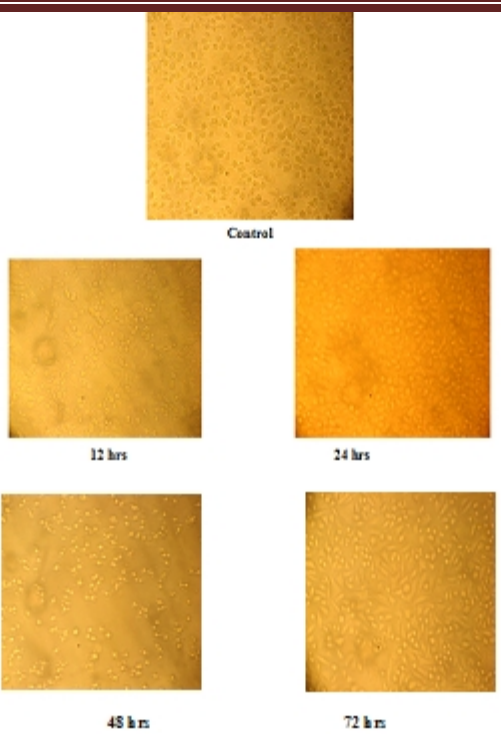

Figure 6(ii). Confocal images of CT3 cells after f-MWCNTs-COOH control (a) 12 hrs (b) 24 hrs and (c) 48 hrs (d) 72 hrs

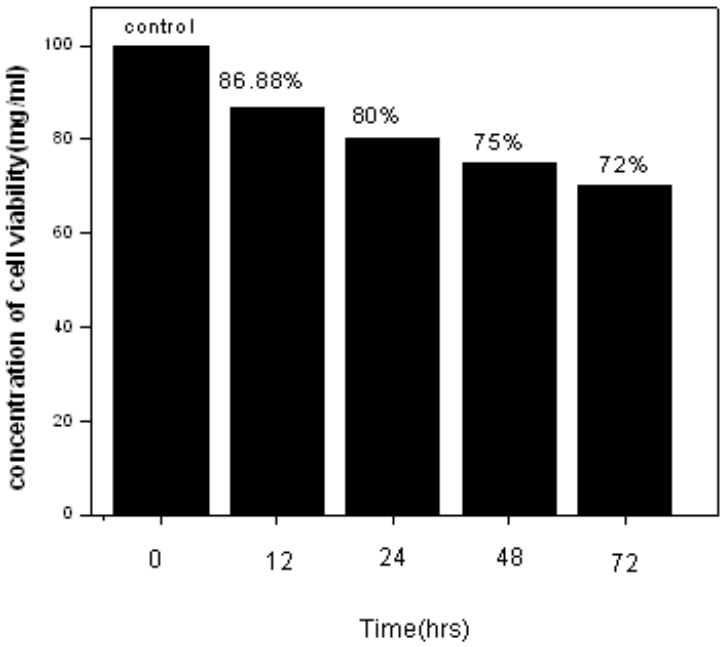

Figure 6(ii).MTT analysis of cell viability (*f*-MWCNTs-COOH)

Source of support: Nil, Conflict of interest: None Declared