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Cytotoxic, genetic and statistical analytical evaluation of functionalized CNTs with C2C12 cells

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ABSTRACT

The present work was designed to apprehend the cytotoxic and genetic evaluation study of multiwalled carbon nanotubes (MWCNTs). The CNTs were surface functionalized via HNO_3 and H_2SO_4 digestion process, confirmed via FTIR spectroscopy whereas the structure of CNTs was visualized with TEM. The obtained CNTs structures were utilized to control the proliferation rate of C2C12 cells. The cells were cultivated and % viabile and non viable cells % were checked by MTT assay with the influence of dose-dependent (500 ng/mL, 1000 ng/mL, 100 μg/mL) CNTs. Further the density of live and dead cells in liquid was also clearifies via confocal laser scanning microscopy (CLSM). Containing these, the genetic study to know the apoptosis caused due to CNTs were checked in presence of caspases 3 and 7 genes. The up-regulation data clearly favours the CNTs are responsible in cells death in C2C12 with the treatment at low (500, ng/mL) and (1000 ng/mL) high dosese. The statistical studies were also performed to authenticate the data for CNTs with different optimized parameters such as detection limit at low concentrations level, recoveries and relative standard deviation at measures 95% confidence level. The analyses palyses a significant role on C2C12 cells with the large effect of CNTs.

1. Introduction

The carbon nanotubes (CNTs), which exhibit unique physico-chemical properties (electrical, mechanical, electro-mechanical etc) attract the scientific community because of their versatile and innumerable applications such as nanoelectronics [1–3], sensors, electro chemical [4,5], field emission [6], X-ray generation [7], nanotweezers [8], chemical force microscopy (CFM) [9,10], storage of energy etc [11]. The CNTs, which is derived from the carbon and are categorized as a single walled (SW) and multi-walled carbon nanotube (MWCNTs) depends upon their single and multiwalled graphene sheets organizations. Due to their very small size and shape it can be utilized as an electrode material to detect the signals for various biological entities [12,13]. The CNTs has recently been utilized for the biomedical applications in various fields such as drug delivery [14], DNA interaction [15,16], toxicity studies [17]. The small dimension facilitates the ability to enter easily into bacteria [18], protein [19], cell membranes either through endocytosis or surface passivation processes [17]. Numerous physicochemical parameters such as size (diameter and length), surface area, concentration of particles, agglomeration, suspension, dispersion, used chemical as catalyst in the cells solution are influenced on toxicity of cells with nanomaterials [20–28]. The toxicity of the CNTs with cells happens when it comes in contact, which depends upon their length, aspect ratios etc [29]. Towards this direction, Poland et al. describes that the length-dependent toxicity in against the peritoneal mesothelium and found that the CNTs have ability to induce inflammation [29]. The thinner CNTs have ability to enter the cells same as asbestos [29], Wang et al. explained that higher toxicity happens of the short length (1–5 μm) CNTs, and have the ~40 to 100 nm, as compared to a sample with a diameter of 10–20 nm to alveolar macrophages [30]. Chatterjee et al. showed the cytotoxic effects against human bronchial epithelial cells (BEAS-2B cells) and human hepatoma cells (HepG2 cells), as model systems with different sized CNTs. Both cells (BEAS-2B and HepG2) were used as target lung organ and drug delivery carriers are possible accumulation in the liver system [31]. Jackson et al. used 15 different types

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of CNTs with nanomaterials and showed the toxicity against mouse lung epithelial cell lines [32]. Very limited information is available in the literature, which reveals that the CNTs exhibit ability to work as toxic effects under *in vivo* [33,34] and *in vitro* [35,36] arrangement. The literature survey reveals that CNTs have been utilized for various purposes in cytological studies for different cells such Patlolla et al. shows that the CNTs plays an important role in normal human dermal fibroblast cells (NHDF) for the cytotoxic induction, genotoxicity and apoptosis [37]. In another report published by Kumarathasan et al. describes that the applicability of different shaped CNTs against human lung epithelial cells (A549) and murine macrophages (J774) [38]. In recent work raw and functionalized CNTs were utilized to control the growth rate in human breast cancer (MCF-7) cells [17]. The composite of SWCNTs and polyami doamine dendrimers (SWCNTs-PAMAM) have been utilized on the C2C12 murine cell line, which indicates that it reduces the growth and DNA damage in C2C12 cells [39]. In another report, single and multiwalled CNTs with pristine graphene flakes were used to evaluate the cytotoxic potential against human dermal fibroblasts (HDFs) and compared with the L-929 fibroblast cell line [40]. In another report, the cytotoxicity of CNTs were evaluated against human lymphocytes *in vitro* condition, at lower doses and it didn't any cause on cell death directly, whether it enriched lymphocyte-mediated cytotoxicity against manifold human cell lines. The CNTs increased the IFN-c, TNF-a and upregulated NF-kB expression by the lymphocytes [41]. The commercially purchased hydroxylated functionalized MWCNTs (MWCNT-OH) were utilized to check the cytotoxicity against human lung epithelial cells (A549) and human alveolar epithelial cell line and found the genotoxic effects and early loss of membrane integrity through MWCNTs [42,43]. In this continuation, Elgrabli et al. reported that CNTs when dispersed in bovine serum albumin (BSA) exhibit reduced toxic effect [44]. Over various cytological studies with CNTs to show the potential of rope like nanotubes against to control the rate of proliferation of cancer cells. Numerous toxicological studies against cells lines in both *in vitro* and *in vivo* conditions, very limited information is available to know the role of CNTs against myoblast cancer cells of C2C12 cells and their genetic studies.

The objective of this work was to examine the cytotoxic potential of functionalized multi-walled carbon nanotubes (MWCNTs), which are not explored yet *in vitro* response for the C2C12 cells. The commercially purchased CNTs were purified and functionalized with acid digestion process and characterized. The surface binding process requires to be functionalized with a variety of reactive groups, which are generally linked to the surface through organic chains. The cytotoxic response or % cell viability due to CNTs was analyzed via MTT assay, whereas the morphological changes in cells were studies via microscopy. The obtained data was validated from the statistical analytical parameters on the basis of ICH guideline which maintain standardizes quality, quantity of chemical and exposed chemical activities for control nanomaterial at minute altitude.

2. Material and methods

2.1. Experimental

2.1.1. (a) Purification and surface functionalization of carbon nanotubes

The multiwalled carbon nanotubes ($MWCNT_S$) were purchased from Aldrich Chemical Co., Ltd U.S.A, and were purified with double distilled water several time for the removal unwanted raw carbon particles and further utilized for acid treatment process. The surface functionalization process was accomplished as previously reported published work [15–17]. After the complete functionalization, CNTs were dried at 60°C in an oven for overnight and examined under their chemical, structural and biological interpretations [15–17].

2.1.2. (b) Characterization of carbon nanotubes

The CNTs were analyzed via Fourier-transform infrared spectroscopy (FTIR) ranges from 4000–400 cm^{-1} to know the surface functionalization and chemical bonding of CNTs, with using KBr pellets. The morphological examinations of black powder material were carried out by scanning electron microscopy (Jeol, JED-2200 series, Japan), transmission electron microscopy (TEM, JEOL JEM 2010 at 200 kV, Japan). The SEM of raw and acid digested/processed CNTs were coated on black carbon tape and this tape was fixed on a sample holder and analyzed at room temperature, whereas CNTs structure were further accessed from TEM. For this, samples were sonicated in an ethanol (EtOH) for ~10–15 min. In this blackish solution, the carbon-coated copper grid (size~ 400 mesh) was inserted for about fraction of second and then removed it, dried at room temperature, then after copper grid was fixed to a sample holder and analyzed.

2.2. Cell culture

The cells (C2C12) were purchased from American Type Culture collection (ATCC-CRL 1772; Bethesda, MD) and cultured in growth medium (Dulbecco's Modified Eagle's Medium, DMEM) with ~10% fetal bovine serum (FBS), 100IU/mL penicillin, and 100 μg/mL streptomycin) in an incubator with humidified environment at 37 °C with 5% CO_2 and 95% O_2. The medium was changed each alternate day and the cells were subcultured after reached 50–60% confluence.

2.3. Cell viability by MTT assay

The viability of cells was tested by the cell culture kit I (MTT) (Cat No.11465007 001, ROCHE, Ltd, U.S.A) with instructions. Briefly, the cell lines were cultured into 96-well plates at 5×10^3 cell/well and incubated the plate for overnight at 37 °C in a an incubator with 5% CO_2. The C2C12 cells were incubated with different concentration (500 ng/mL, 1000 ng/mL, and 100 μg/mL) of CNTs and transferred to the incubator for 24 h, 48 h and 72 h. The cytotoxic assessment or mitochondrial dysfunction was evaluated by the use of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-di phenyltetrazolium bromide, a tetrazole, 10–20 μL to each well) incubated for about 4 h. After the complete incubation, the samples were treated with solublizing buffer (DMSO, 100μL/well), a purple colour was appeared at this stage and deepen over pipeting. In this condition, the MTT salt was reduced and form a purple formazan in the mitochondria of living cells. The obtained coloured solution, absorbance was measured via Elisa Reader (Bio Rad) at 570 nm. The percentage (%) viability was calculated as follows:

$$\% \text{ Viability} = \text{OD (optical densities) in sample well/OD in control well} \times 100$$

2.4. Confocal scanning laser microscopic (CSLM) study

For the quantitative and morphological evaluation, the cells were cultured in a confocal disk (CD), including different concentration of CNTs and were incubated in an incubator for overnight at 37 °C. As the cells confluence was reached above 50–60%, cells medium was discarded from the CD and washed with PBS. After this, cells were fixed with 70% alcohol (EtOH) and to keep for

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