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Comparative *in vitro* cytotoxicity study of carbon nanotubes and titania nanostructures on human lung epithelial cells

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ABSTRACT

The aim of this study is to assess *in vitro* cytotoxic effects of titania nanostructures and carbon nanotubes (CNTs) by exposing A549 lung epithelial cell line to these materials. Titania nanotubes (TiNTs) were grown by hydrothermal treatment of TiO_2 nanoparticles, followed by annealing them at 400 °C. The titania nanostructures obtained on annealing (mixture of nanotubes and nanorods) were hollow and open ended, containing 3–5 layers of titania sheets, with an internal diameter ~3–5 nm and external diameter ~8–10 nm, and a specific surface area of 265 m^2/g. As-supplied single walled (SWCNTs) and microwave plasma enhanced chemical vapour deposition (MPCVD) grown multi walled carbon nanotubes (MWCNTs) were used in this study. The lengths and diameters of the SWCNTs were 5–10 nm and 0.5–3 nm respectively. The lengths and diameters of the MWCNTs were 25–30 μm and 10–30 nm respectively. The cell viability was evaluated using the MTT (3-(4,-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium) assay. No significant cytotoxic effects of titania nanostructures were observed over a period of a week of testing time, while the presence of CNTs in some cases demonstrated significant cytotoxic effects. Finally, possible reason of cytotoxicity is discussed in the light of microstructures of materials.

1. Introduction

The use of engineered nanomaterials has raised concerns about the unknown toxic effects of these materials due to increased environmental exposure [1,2]. Nanocrystalline titania (TiO_2) is an important material which is utilised in a wide range of applications including photocatalysis, self-cleaning glass, low cost solar cells [3]. Carbon nanotubes (CNT), both single walled (SWCNT) and multi walled (MWCNT) form another class of unique nanomaterials. Over the last two decades, CNTs have attracted great attention due to their remarkable mechanical, electronic and chemical properties, which are utilised in various applications [4–6]. Airborne nanomaterials can be inhaled and cause damage to the alveolar compartment of the respiratory tract. Results have shown that nanomaterials are able to rapidly enter into cells, and distribute in the cytoplasm and intracellular vesicles. Therefore, comprehensive studies concerning the cytotoxic effects of these new generation materials is warranted, prior to their widespread use.

A few studies have reported concerns about cytotoxic effects of carbon nanotubes, and the toxicity was found to be irrespective of length and the presence of metallic impurities [7,8]. Literature published on carbon nanotubes [7], titania nanoparticles [9] and other nanoparticles [10], has revealed a correlation between the different physical properties of the materials with their toxic effects. There are only a few reports available on hydrothermally synthesised titanate nanofilaments so far [11,12] which have concluded that nanostructures are cytotoxic. More research to evaluate the potential cytotoxic effects of titania nanomaterials and carbon nanotubes on cells is needed.

In this work, we aim to compare the *in vitro* cytotoxic effects of hydrothermally synthesised titania nanostructures and multi walled carbon nanotubes (synthesised in-house by microwave plasma chemical vapour deposition (MPCVD) [13,14]) on A549 lung epithelial cell line. Microstructural properties of these nanomaterials were also studied before exposing them to lung epithelial cells. The results show that titania nanostructures are not cytotoxic, within the range of time period considered in the study. Conversely, carbon nanotubes show significant cytotoxicity for lung epithelial cells within the same time-frame.

2. Experimental methods

2.1. Synthesis of titania nanotubes

Titania nanotubes were synthesised by the hydrothermal treatment of a suspension of 0.8 g *Degussa* P25 (now known as Evonik

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Aeroxide TiO_2 P25) nanoparticles (roughly 70% anatase and 30% rutile, crystallite size ~23 nm, specific surface area = 51 m^2/g) in 9.5 M NaOH aqueous solution at 120 °C for 24 h. The product was then washed thoroughly with 0.1 M HCl solution followed by distilled water washing and dried in air at room temperature [15]. The white powder obtained was annealed in air at 400 °C for 2 h at a ramp rate of 5 °C/min. The annealed sample was characterised using high resolution electron microscopy (HRTEM) and X-ray diffraction (XRD).

2.2. Synthesis of carbon nanotubes

SWCNTs were purchased from carbolex Inc. Ltd. US, while MWCNTs were grown on Si substrate using MPCVD method [13]. The as-purchased SWCNTs were ~99% pure with < 1% of impurities consisting of the catalyst (Fe in this case) while MWCNTs contained < 1% of Cobalt (Co) as metallic impurities. For MPCVD growth of CNTs, the catalyst used was a 3 nm Cobalt (Co) thin film. The cobalt thin film catalyst was deposited on the substrate using dc magnetron co-sputter deposition at a power of 50 W and an Ar pressure of 1×10^{-2} Torr. Prior to the CNT growth, the catalyst film was etched using argon plasma for 3 min. The gas pressure was 23.2 mbar and the microwave power was 300 W. The growth time was 5 min. The gas pressure during the growth was kept constant at 40 and 10 sccm of nitrogen and methane respectively [14]. The as-deposited CNTs were analyzed using FEI Quanta 200 3D scanning electron microscopes (SEM). A micro Raman spectrometer, with a laser source of wavelength of 633 nm was used to determine the quality of CNTs.

2.3. Samples for cytotoxicity studies

Three different concentrations *i.e.* 0.1, 0.5 and 1.1 mg/mL of titania nanostructures were prepared in phosphate buffer saline (PBS). For CNTs, solutions used were 10 times less concentrated than titania nanoparticles. Therefore, the CNT solutions used were 0.01, 0.05 and 0.11 mg/mL in PBS. All samples were sterilised at 121 °C for 20 min in a pressurised steam autoclave (Proirclave, UK) and ultrasonicated for 1 min prior to cell culture analysis.

2.4. Cell culture technique

A549 human lung adenocarcinoma cell line obtained from ATCC (American Type Culture Collection; Rockville, MD) was used for the biological evaluation of the test substrates. Cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% foetal calf serum in 75 cm^3 Nunc tissue culture flasks. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and were sub-cultured when they reached confluence using 0.25% – Trypsin ethylene diamine (EDTA) to provide adequate numbers of cells for the various *in vitro* culture studies. A suspension of 2×10^4 cells were added to each well of 96-well plates and allowed to adhere during incubation at 37 °C in 5% CO_2 for 3 h. A 20 μL aliquot of the 0.1 mg/mL of titania nanostructures, SWCNTs and MWCNTs were added to each well (to give a final concentration of 10 μg/mL) and replaced in the incubator.

2.5. Cell viability study

Cell viability was determined after 1, 3 and 7 days in culture using the standard colorimetric MTT assay. A commercial MTT assay kit (Sigma–Aldrich, UK) was used, employing a modification of Mosmann method [16]. The MTT assay reagent was prepared as a 5 mg/mL stock solution in PBS, sterilized by Millipore filtration, and stored in the dark. At the appropriate time-points, MTT stock solution (10% of total volume) was added to each well. After 3 h incubation at 37 °C in 5% CO_2, the reagent was aspirated and 200 μL of MTT solvent (Sigma–Aldrich, UK) was added to dissolve the formazan crystals. The solution was agitated homogeneously for 15 min on a shaker to ensure adequate dissolution. The optical density of the formazan solutions was read by spectrophotometer on an ELISA plate reader (Tecan Sunrise, Tecan Austria) at 570 nm with the background absorbance value measured at 650 nm. The absorbance values recorded were considered to be proportional to the number of viable cells in each sample well.Statistical analysis

All data reported here are expressed as mean ± standard deviation values. A one-way analysis of variance (ANOVA) was applied to test for statistically significant differences between the sample types, where a *P*-value of $P < 0.05$ is considered to be statistically significant. A post-hoc pair-wise Dunnett's Multiple Comparison Test was applied to compare values between all the sample types against the PBS treated control wells to ascertain differences in the number of viable cells between samples. All statistical analysis was performed using GraphPad Prism Version 9 for Windows.

3. Results and discussion

3.1. Characterisation of titania and carbon nanotubes

Fig. 1(a) and (b) represent HRTEM images of titania nanostructured sample and P25 nanoparticles. HRTEM studies revealed

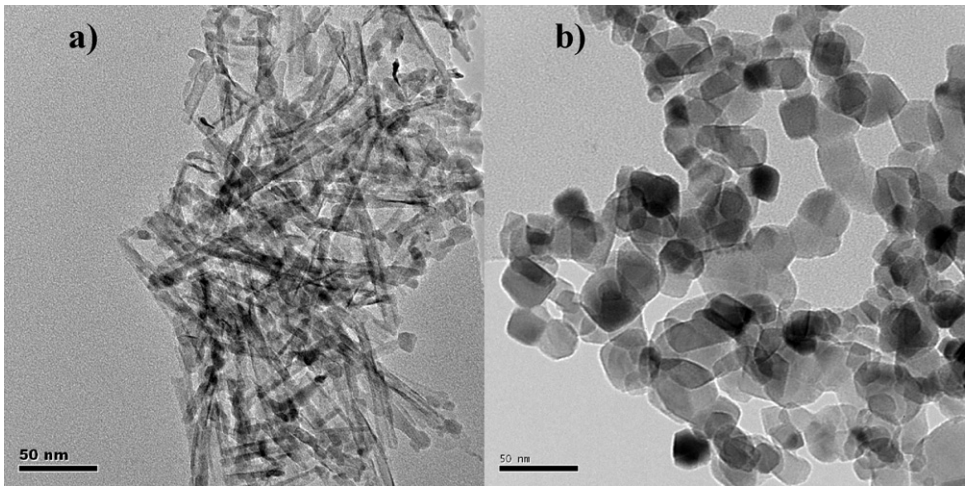

Fig. 1. TEM images of (a) TiNT and (b) P25 nanoparticles (scale bar 50 nm).

that annealed sample contained an aggregated mixture of 30% nanotubes and 70% rods. Hereafter, a mixture of nanotubes and nanorods obtained after annealing at 400 °C will be referred as nanotubes (TiNT) for the sake of simplicity. The nanotubes (Fig. 1(a)) were found to be asymmetrically multi-walled with 3 layers on one side and 5 on the other. The internal diameter was determined to be 5 nm and external diameter was 8–10 nm. P25 nanoparticles (Fig. 1(b)) were found to be aggregates of particles of crystallite size ~23 nm (determined from XRD).

The crystalline structure of the titania nanostructures was investigated using X-ray diffraction measurements. XRD patterns of titania samples are shown in Fig. 2. From XRD analysis, it was confirmed that the nanotube sample contained anatase phase ($a = b = 3.79$ Å, and $c = 9.51$ Å, JCPDS 21-1272) with no evidence of rutile or amorphous phase. P25 particles are known to possess roughly 70% anatase and 30% rutile. The crystallite size was determined using Scherrer equation considering the most intense X-ray diffraction peak.

The SEM image of vertically aligned MWCNTs is shown in Fig. 3(a). The height of the CNT films ranging from 25–30 μm. The diameters of the nanotubes were estimated to be in the range of 10–30 nm with few walls, and the interspacing between the walls was found to be 0.34 nm. TEM images of MWCNTs are shown in Fig. 3(b). The bamboo-shaped CNTs are obtained and are shown in the TEM image. Fig. 3(c) shows the SEM images of SWCNTs. As said earlier, these CNTs were purchased in powder form and then dispersed in ethanol. A drop of this dispersed solution was put onto silicon substrate for SEM. The SEM analysis shows a bundle of SWCNTs. Detailed TEM analysis showed their length to be in the range of 5–10 nm with diameter of 0.5–3 nm (Fig. 3(d)). Raman studies (not given) on the CNTs verified their qualitative microstructure and purity.

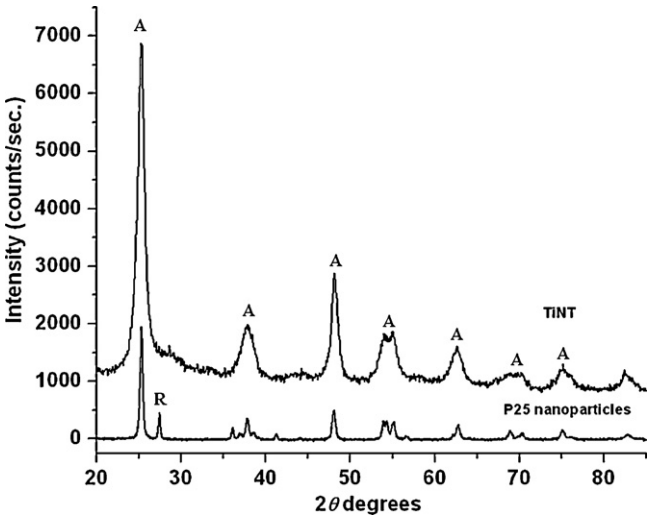

Fig. 2. X-ray diffraction pattern of titania nanotubes and nanoparticles. A = anatase; R = rutile.

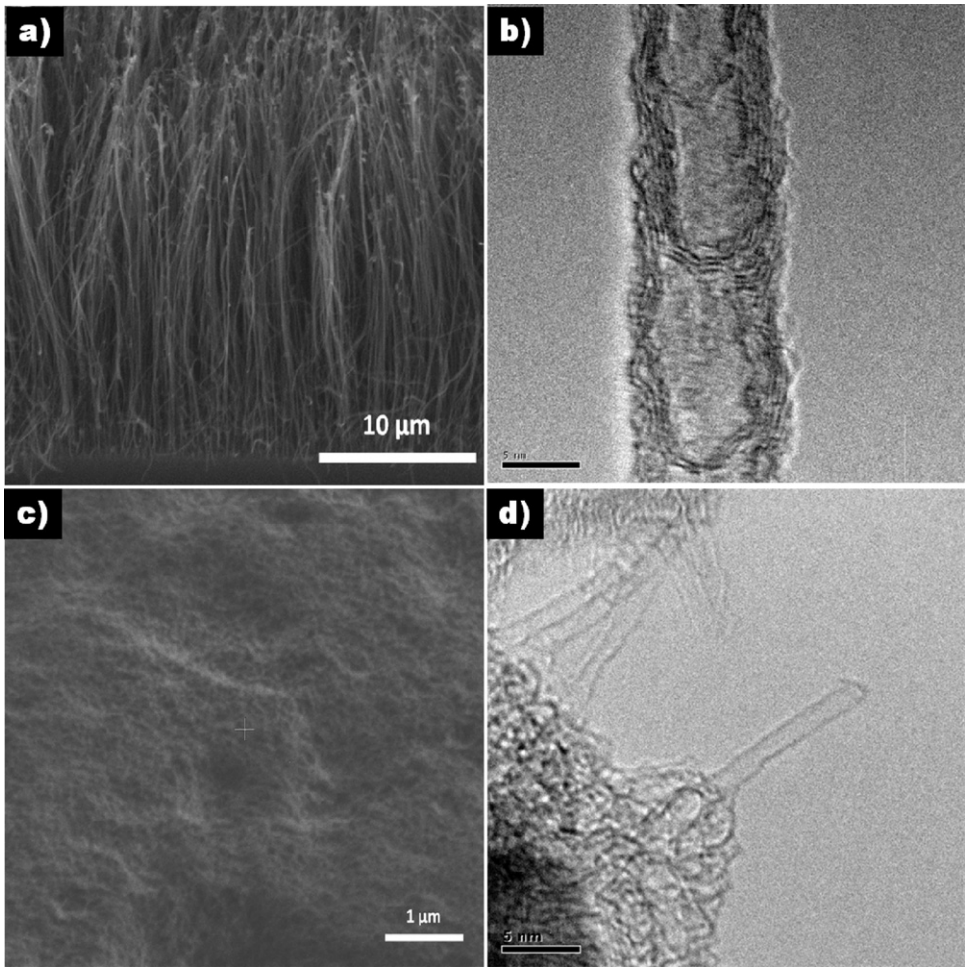

Fig. 3. SEM images of CNTs ((a) and (c)) of as produced vertically aligned MWCNTs on Si and SWCNT as purchased from carbolex inc. ((b) and (d)) TEM of a MWCNT and SWCNT strand (scale bar 5 nm).

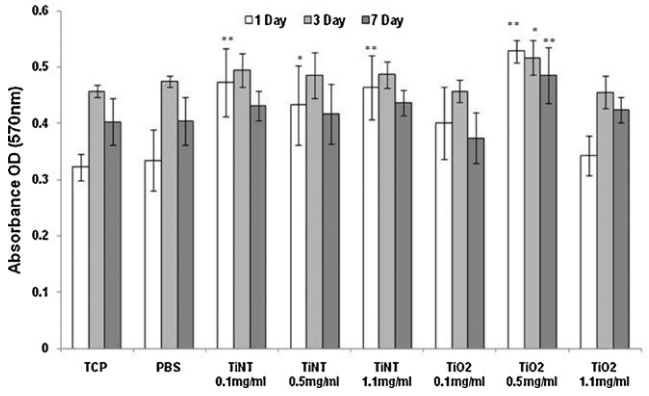

Fig. 4. Optical density measurements for MTT assay of cell viability of A549 cells treated with TiNTs and TiO_2 particles after 1, 3 and 7 days in culture. (*) indicates a significant increase in absorbance value ($P \leq 0.05$), (**) indicates a significant increase in absorbance ($P \leq 0.01$) as compared to the controls.

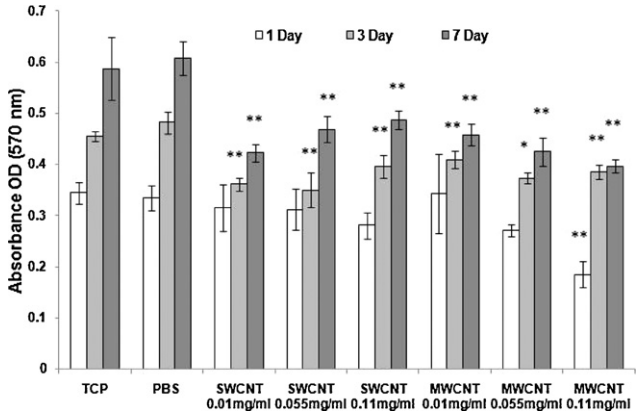

Fig. 5. Optical density measurements for MTT assay of cell viability of A549 cells treated with SWCNT and MWCNT particles after 1, 3 and 7 days in culture. (*) indicates a significant decrease in absorbance value ($P \leq 0.05$), (**) indicates a significant decrease in absorbance ($P \leq 0.01$) as compared to the controls.

3.2. Cell viability analysis

The nature of the affects of adding solutions of SWNTs, MWNTs, TiO_2 nanoparticles and TiNTs to A549 cultures was investigated using an MTT assay over a period of 7 days incubation. This assay quantifies the cell viability through the conversion of the MTT reagent to formazan crystals, which are subsequently solubilised and the concentration determined by spectrometry. Two control samples were also assessed, one of which had nothing added into the wells (designated tissue culture plastic – TCP) and the other one had 20 μL of PBS (the carrying solution for both types of nanotubes) added into the media. For comparative statistical analysis, all samples were compared to the PBS treated wells. Fig. 4 shows the relative amounts of viable cells in each well after the addition of TiNTs and TiO_2 nanoparticles. After 24 h in culture, there was an increase in cell viability across all the samples treatments compared to the PBS control well. There was a statistically significant increase in viable cell numbers in the wells treated with 0.5 mg/mL TiNT ($P \leq 0.05$) and those treated with 0.1 and 1.1 mg/mL TiNTs ($P \leq 0.01$). The greatest increase in cell number was observed from the cells treated with 0.5 mg/mL TiO_2 solutions ($P \leq 0.01$). After 3 days, in culture the only statistically significant difference in cell viability was seen between the control well and the 0.5 mg/mL TiO_2 ($P \leq 0.05$) treated well. There was no significant increase or decrease in cell number across any of the other treated cells at this sampling point. Furthermore, following 7 days of exposure to various concentrations of titania solutions, there was neither significant increase nor decrease in cell viability.

All these results point towards the fact that titania nanotubes and nanoparticles do not show significant detrimental effects on the viability of the A549 lung epithelial cells used in the study over a one week incubation. This is in agreement with some published reports indicating that titania nanotubes do not cause chronic cytotoxic effects [17]. Despite having a greater aspect ratio, and hence 8 times higher surface area than P25 nanoparticles, titania nanotubes demonstrated similar cell viability to nanoparticles. Careful inspection of Fig. 4 also revealed larger absorbances for all titania samples (in general) in comparison to the positive control (TCP) and phosphate buffer solution (PBS). It has previously been reported that titania nanoparticles enhance bone cell adhesion and proliferation [18]. It has been argued that titania nanostructures tend to aggregate in solution and these aggregates are not disintegrated by lung surfactants [19]. A previous report also mentions the formation of complex secondary particles by the interaction of these aggregates with the cell culture medium components [20]. This in part may possibly alter the active surface area of titania nanostructures. Therefore, it is probable that due to the binding of nanoparticles with cell culture medium components and alteration in surface area, the nanoparticles are unable to interact with the cell organelles effectively, hence remain inactive inside the cell, even if titania nanoparticles are taken up by cells. Another possibility is that the internalisation of aggregated nanoparticles is hindered. Non-toxic titania nanotube aggregates, possessing very high surface area, may provide a suitable material for bone implants or other biomaterial applications. Nano-grain surface properties and high surface area may also provide greater interaction of proteins and enhance osteoblast activity. Furthermore, the porous channelled structure of nanotubes and open spaces between nanotubes may allow sustained flow of blood and nutrients after the attachment of osteoblasts on nanotube surface [21]. Reports have also demonstrated that titania nanotubes provide favourable templates for growth and maintenance of bone cells [17]. It is possible that similar growth effects are being observed with this cell line due to the addition of the titania nanoparticles.

Fig. 5 shows the relative amounts of viable cells in each well after the addition of various SWNT and MWNT solutions over 7 days. After 24 h in culture there was no significant decrease in cell viability across the various sample types, except for the 0.11 mg/mL MWCNT treated cells ($P \leq 0.01$). There was a statistically significant decrease ($P \leq 0.01$) in the number of viable cells across all the treated wells after 3 days incubation. The same trend ensued after 7 days *in vitro* studies, where again there was a statistically significant decrease in cellular activity ($P \leq 0.01$) in comparison to the PBS control well. SWCNTs demostrated similar and insignificant cytotoxicity at all doses after 1 day of treatment while in case of MWCNTs, the dose dependence is clearly noticeable, the highest concentration being the most significantly cytotoxic. Comparing the dose dependence over all 7 days of exposure, SWCNTs appear to be least cytotoxic at higher concentrations *i.e.* 0.11 mg/mL while MWCNTs are more cytotoxic at higher concentrations. This correlates well with other dose dependent studies where MWCNTs were found to be more toxic at higher concentration of 50 μg/mL [22].

In order to guage the relative affects of the TiNT, SWCNT, MWCNT particles at the same concentration on the viability of A549 cells, 0.1 mg/mL of each type of solution was assessed over 7 days of incubation (Fig. 6). After 1 day there was a statistically significant increase ($P \leq 0.01$) in cell viability in the wells treated with the TiNT solutions, compared to the PBS control. There was no significant difference between the SWCNT treated cells however, there was a statistically significant decrease in cellular activity in the wells treated with the MWCNTs *in vitro* ($P \leq 0.01$). At the 3 day sampling point, there was no significant increase or decrease in the

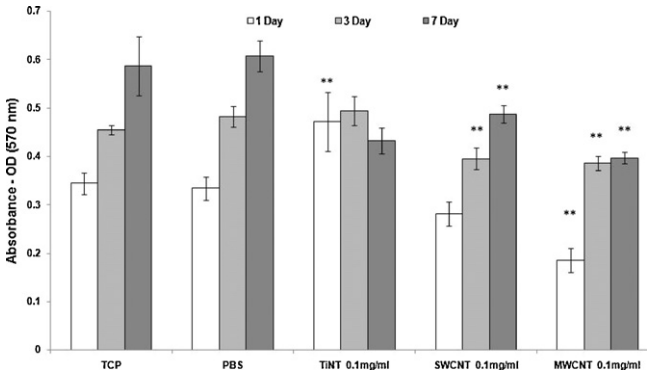

Fig. 6. Optical density measurements for MTT assay of cell viability of A549 cells treated with TiNT and MWCNT particles with concenration 0.1 mg/ml after 1, 3 and 7 days in culture. (**) indicates a significant decrease or increase in absorbance ($P \leq 0.01$) as compared to the controls.

TiNT treated well whereas a decrease in cell viability was detected in both the SWCNT and MWCNT treated wells ($P \leq 0.01$). Finally, at the final sampling point (day 7) the same trend was observed where there was no statistical difference between the TiNT treated wells and the PBS control well, but there was a statistically significant decrease in cell viability on the SWCNT and the MWCNT treated cells ($P \leq 0.01$).

Casey et al. reported that there may be indirect toxicity associated with SWCNTs where the removal of SWCNT and associated impurities from conditioned medium by centrifugation and filtration resulted in an alteration composition of the medium [23]. They argued that the principal effect is one of reduced cell proliferative capacity rather than viability. According to their report, the reduction in cellular activity is not inherently due to the toxicity of the nanomaterials. Similar absorptive interactions of nanomaterials on culture media have been reported. Once again cellular effects were higher for the nanoparticle with the larger surface area. On the contrary, if indirect toxicity of CNTs due to interaction with cell culture media is considered to be the primary source of toxicity, there would be fair chances of similar or closely related cytotoxic effects of both types of CNTs. However, Fig. 6 clearly indicates that MWCNT shows relatively higher cytotoxic effects when compared with SWCNT after 1 day of treatment. This may be due to the difference in the defects and impurity content (such as soot, amorphous carbon, carbon black, catalyst, etc.) present in the MWCNTs. As purchased SWCNTs were ~99% pure with trace amount of Fe as impurity while laboratory synthesised MWCNTs contained impurities including carbonaceous content and trace amounts of Co. These results are consistent with other published reports [24]. Wick et al. found CNT sample containing highest carbonaceous content to be more toxic than other samples [25]. Similarly, there is evidence that the presence of Co as impurity can cause chromosome loss at doses compatible with the MWCNT Co content used in the study [22].

4. Conclusions

In vitro cytotoxic effects of titania nanotubes, titania nanoparticles and carbon nanotubes were investigated by exposing them to the A549 lung epithelial cell line, over one week *in vitro*. The presence of titania nanostructures showed an increase in cell viability compared to the control wells at almost all doses, while the presence of CNTs in some cases demonstrated significant cytotoxic effects. The cell culture analysis here suggests that hydrothermally synthesised titania nanotubes are non-cytotoxic. MWCNTs were more cytotoxic compared to SWCNTs when exposed to the A549 cell line, particularly at the lowest concentration of 0.1 mg/mL. Further research is needed to understand the mechanisms involved in the toxicity and fate of nanomaterials before their widespread application in commercial products.

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