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Cytotoxicity and genotoxicity of multi-walled carbon nanotubes with human ocular cells

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Cytotoxicity and genotoxicity of plasma-modified multi-walled carbon nanotubes (MWCNTs), including hydroxyl-MWCNTs (MWCNT-OH), carboxyl-MWCNTs (MWCNT-COOH) and pristine MWCNTs, with human ocular cells (e.g. retinal pigment epithelium (RPE) cells) have been studied in this work. The addition of MWCNT-based materials caused few change in cell morphology while the presence of MWCNTs was observed inside the cells using transmission electron microscopy (TEM), suggesting possibility of MWCNTs passing through the cell membranes without damaging cells. Cell viability measurements suggested that MWCNT-COOH exhibited better biocompatibility than other MWCNT materials studied in this work. Lactate Dehydrogenase (LDH) release level was found to be less than 30% with all types of MWCNT-based materials. Reactive Oxygen Species (ROS) generation was visible but not severe with addition of nanotubes. A smaller oxidative stress level was obtained from MWCNT-COOH. Cell apoptosis was found to be less than 1.5% with addition of MWCNT-based materials. Particularly MWCNTs were found to be swallowed by cells and released by cells after 72 h without damaging cells, which may be considered as a potential vector for ocular genetic diseases. Plasma modification of MWCNTs particularly with –COOH was found to be an efficient way to improve ocular biocompatibility of MWCNTs, suggesting a fast and useful way to modify MWCNTs for applications in areas such as biology and biomedicine.

multi-walled carbon nanotubes, ocular biocompatibility, retinal pigment epithelium (RPE) cells, cytotoxicity, genotoxicity

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Carbon nanotubes (CNTs), including single-walled carbon nanotubes (SWCNTs) and MWCNTs, have attracted much interest since Iijima [1,2] first reported in 1991. Since that time CNTs have been studied in a range of diverse applications including biological, chemical and electrical [3–8] owing to their unique mechanical, electronic and thermal properties [9,10]. Of particular interest, applications of nanomaterials particularly CNTs in biological areas such as drug delivery, controlled release, and biosensors have attracted much attention [11–13]. For commercial utilization of CNTs, it is essential to evaluate the influence of CNTs on environment and human health [14,15]. Compared with SWCNTs, MWCNTs have great advantages on practical biomedicine applications considering their more simple preparation process, cheaper price and larger industry production scale. Bellucci et al. [16] found that MWCNT bucky paper could influence the growth of some cancer cells such as colorectal, breast and leukemic while introduction of MWCNTs caused no change on human arterial smooth muscle cells and normal human dermal fibroblast cells (NHDF). They also found that injection of MWCNTs into rats induced a moderate inflamma-

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tory reaction but has no mutagentic effects. Bellucci et al.'s results showed that MWCNTs were low toxic. More recently, however, scientists reported that MWCNTs were very toxic. For instance, Patlolla et al. [17] reported that MWCNTs were very toxic to NHDF. They found that MWNT induced massive loss of cell viability through DNA damage and programmed cell-death. Inhalation exposure of MWCNTs to rats were found to cause increased lung weights, pronounced multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis [18]. The genotoxicity of MWCNTs have also been investigated in our previous work [19]. We found that MWCNTs could accumulate in mouse embryonic stem (ES) cells and make damage on DNA probably via reactive oxygen species (ROS) generation. Ocular biocompatibility of MWCNTs, however, has not been reported previously. Ocular diseases have been considered as the serious global problems for public health. Efficiency of ocular medicines, however, was limited to a large extent due to presence of the blood-ocular barrier and the cornea barrier. It has been known that MWCNTs can easily pass through the cell membranes, providing great possibility to develop high efficient ocular medicines based on MWCNTs which may be able to pass through the presenting barriers. For ocular biomedical applications of MWCNTs, it is essential to investigate ocular biocompatibility of MWCNTs in details. In addition, exposure of eyes to MWCNTs is unavoidable during ordinary utilization such as delivery, production, research experiments and biomedicine applications. Evaluation of MWCNTs on ocular biocompatibility is necessary and important.

In the present work, we report for the first time on ocular toxicity of MWCNTs with human retinal pigment epithelium (RPE) cells. RPE is a monolayer of cells located close to the neural retina. The integrity and function of RPE are essentially related to neural retina homeostasis and ocular diseases [20]. In this work, ARPE-19, a cell line derived from human RPE, was cultured in MWCNT-containing media for biocompatibility evaluation. Particularly, plasma modified MWCNTs with various functional groups such as carboxyl (MWCNT-COOH) and hydroxyl (MWCNT-OH) have been found to exhibit different toxicity to RPE cells, providing useful information for efficient improvement of ocular toxicity of MWCNTs via functionalization for future applications.

1 Materials and methods

ARPE-19 cells (American Type Culture Collection) were cultured with MWCNT-based materials mixed cell culture medium (see Supporting Information for details). Various types of MWCNTs including pristine MWCNTs, MWCNT-OH, and MWCNT-COOH were used respectively. Cells cultured without the addition of nanotubes were used as the control throughout the work. Though pristine MWCNTs could not dispersed well in cell culture medium, functionalized MWCNTs were found to be dispersed and maintained well.

Cell viability rates with MWCNT-based materials were determined using Cell Counting Kit-8 (CCK-8). Calculation of cell viability assay was shown in Supporting Information. Influence of MWCNTs and derivatives on the ARPE-19 cell membrane integrity was investigated using Lactate Dehydrogenase (LDH) release assay. The experimental details were shown in Supplementary Materials. ROS Assay was employed to study the effect of MWCNT-based materials on oxidative stress. Two different methods including Confocal Laser Microscopy and Fluorescent analysis were used to determine the ROS generation level. In addition, Hoechst Staining Kit was used to quantitatively analyze the apoptotic and necrotic cells.

2 Results and discussion

Presence of various functionalized groups on MWCNTs were confirmed using Raman and Fourier transform infrared (FTIR) spectroscopy as shown in Figure S1. Two characteristic bands due to the G band and D band of MWCNTs were observed in Raman spectra (Figure S1(a)). The intensity ratio of G to D band ($I_{G/D}$) was found to be around 2∶1 for pristine MWCNTs. The introduction of functionalize groups e.g. –OH and –COOH caused decrease in $I_{G/D}$ significantly. The $I_{G/D}$ was found to be 1.5∶1 for MWCNT-OH and 1.6∶1 for MWCNT-COOH, suggesting introduction of defect contents with addition of functionalized groups. FTIR spectra of MWCNTs, MWCNT-OH and MWCNT-COOH were shown in Figure S1(b). The wide band at 3450 cm^{-1} both observed from spectra of MWCNT- OH (red line, Figure S1(b)) and MWCNT-COOH (blue line, Figure S1(b)) is due to the vibration mode of –OH while no such band was found from the pristine MWCNTs (black line, Figure S1(b)), suggesting successful modification of MWCNTs. The band around 1750 cm^{-1} was visible at the spectra of functionalized MWCNTs while it was negligible in the untreated MWCNTs. This kind of band can be attributed to the stretching vibration of carbon-oxygen bonds.

Influence of functionalized MWCNTs on ARPE-19 cell density and morphology were shown in Figure 1. It was found that cells became smaller and rounded after addition of 100 μg/mL MWCNTs over 72 h (Figure 1(b)), suggesting a bit toxicity of MWCNTs with ARPE-19 cells. Agglomeration of MWCNTs was visible around cells. Addition of MWCNT-OH and MWCNT-COOH (Figure 1(c) and (d)), however, exhibited smaller agglomeration and toxicity compared to the pristine MWCNTs.

Figure S2 shows presence of MWCNTs inside the ARPE-19 cell. Presence of MWCNTs inside the cell was

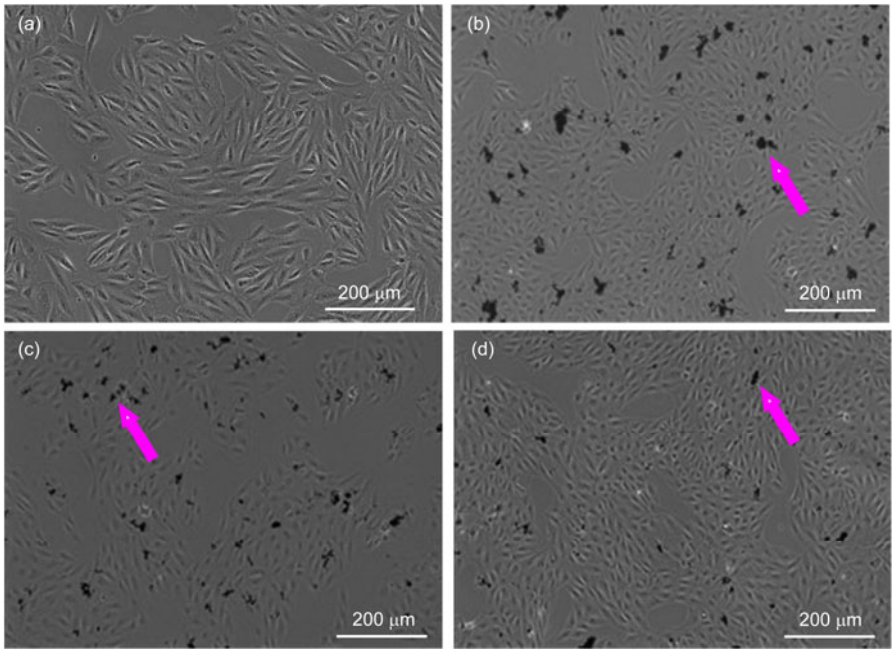

Figure 1 Optical micrograph of ARPE-19 cells incubated (a) without MWCNTs, (b) with MWCNTs, (c) with MWCNT-OH, and (d) with MWCNT-COOH over 72 h. Arrows indicative of MWCNTs and derives.

obvious after incubation with 50 μg/mL MWCNTs over 24 h (Figure S2(a)). But no visible damage was observed in the cell. Liquor bubble was found to be increased and expanded at the edge of cell membrane (Figure S2(a) and Figure 2(b)), but no significant change on cell morphology was observed. As arrows indicated in Figure 2(a), MWCNTs were found to spread apart and ready to release from the cell over 72 h. It may be attributed to penetration of MWCNTs into and out the cytoplasm through the cell membrane by swallowing [21,22].

CCK-8 assay is a kind of popular technique for determination of cell viability *in vitro* toxicology studies [23,24]. As shown in Figure 2(b) and Figure S3, RPE cell viability decreased with time and concentration increasing after introduction of MWCNTs, MWCNT-OH and MWCNT-COOH (P<0.05). It was found that MWCNT-COOH exhibited the lowest toxicity than other two types of MWCNT-based materials while higher cell viability was obtained from MWCNT-OH compared to pristine MWCNTs, suggesting plasma modification may be a potential way to improve biocompatibility of MWCNTs. This might be attributed to the improved dispensability of functionalized MWCNTs which result in lower toxicity. After 72 h incubation, RPE cell viability of MWCNT-COOH induced sample was still higher than 60% even with addition of 100 μg/mL nanomaterials (Figure 2(b)), indicating well biocompatibility of MWCNT-COOH.

Cytotoxicity of MWCNTs and functionalized MWCNTs can be further confirmed using LDH release assay. LDH is a stable cytosolic enzyme released upon cell lysis, indicative of cell membrane integrity and cell toxicity [23]. Figure 2(c) and Figure S4 showed the LDH release level of RPE cells with MWCNT induced. LDH release was found to be dose dependent and time dependent (P<0.05). The lowest LDH release level was obtained at the MWCNT-COOH containing sample when compared with MWCNT-OH and the pristine MWCNTs, suggesting the lowest cytotoxicity of MWCNT-COOH. Similar with the cell viability results, a lower LDH release level was observed at the MWCNT-OH compared to the MWCNTs. Maximum LDH release for the MWCNT-COOH induced was lower than 20% even when 100 μg/mL nanomaterials were exposed over 72 h (Figure 2(c)), suggesting that the carboxyl-functionalized MWCNTs caused very small damage on the RPE cell membrane. These results are well consistent with the cell viability measurement.

ROS Assay is generally used for detection of oxidative stress level in cells. Increased ROS generation is an initiating factor of toxicity in nanomaterials exposed cells [25,26]. Normally ROS generation levels are determined using dichlorofluorescein deacetate (DCFH-DA) [27]. Typically DCFH-DA is de-esterified to dichlorodfluorescein (DCFH) by cellular esterases. The generation of ROS will oxidize DCFH to form fluorescent dichlorofluorescein (DCF). Generation of net intracellular ROS is thus obtained from the increased fluorescence intensity. As shown in Figure 3, representative microphotographs of ROS generation induced by nanotubes were showed in green color. The amounts of ROS generation are in a good consistence with the cell viability and LDH release level measurements. The lowest content of ROS generation was obtained at the MWCNT-COOH containing sample (Figure 3(d)). MWCNT-OH induced lower

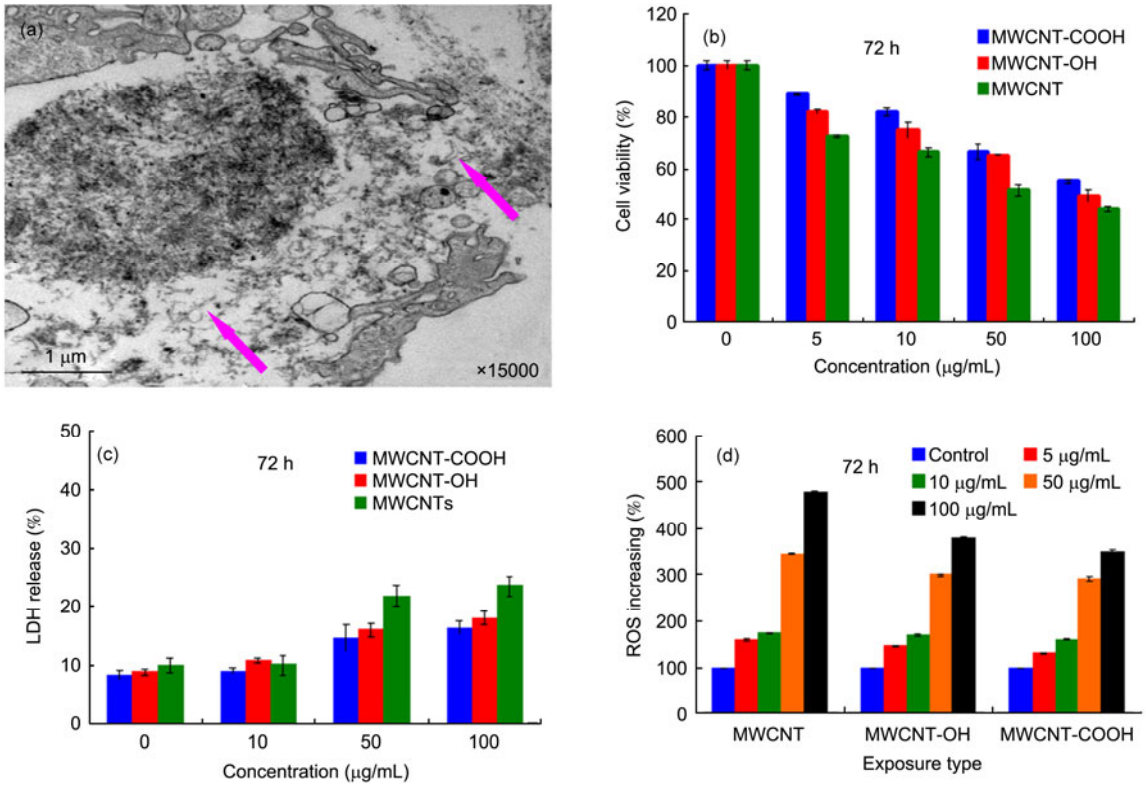

Figure 2 (a) TEM images of ARPE-19 cell after incubation with 50 μg/mL MWCNTs over 72 h. Arrows indicates the release of MWCNTs from cell internal. (b) Cell survival rate assay of ARPE 19 cells incubated with various amount of MWCNT, MWCNT-OH, MWCNT-COOH over 72 h. (c) LDH assay for detection of ARPE-19 cells incubated with MWCNT, MWCNT-OH and MWCNT-COOH over 72 h. (d) Percentage change in ROS generation after exposure of cells to various concentrations of MWCNTs, MWCNT-OH and MWCNT-COOH over 72 h.

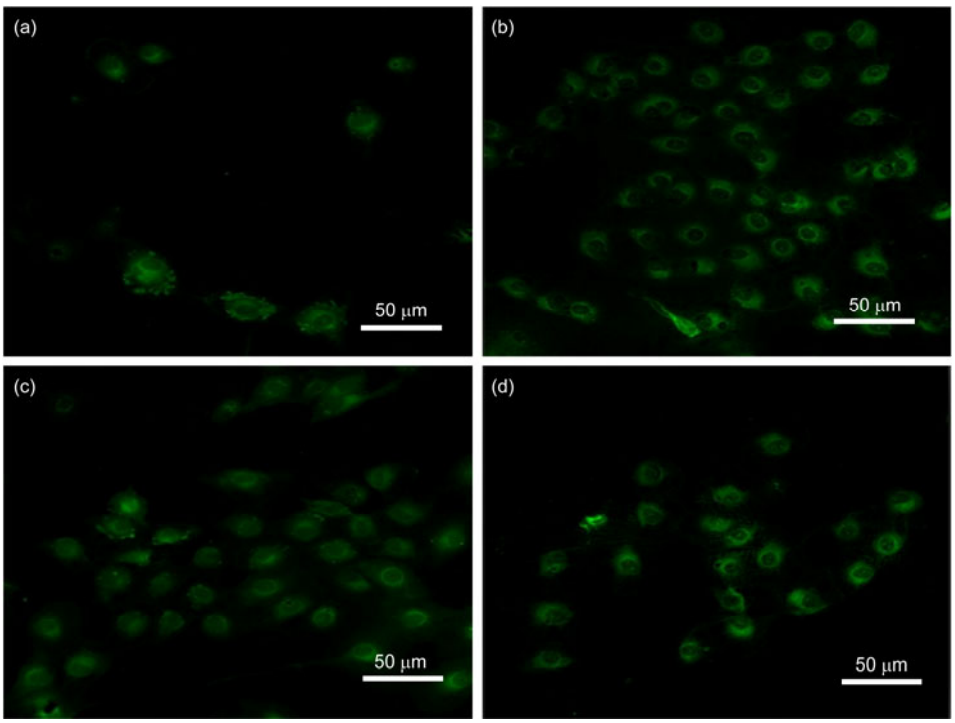

Figure 3 Representative microphotographs of (a) the control, (b) MWCNTs, (c) MWCNT-OH and (d) MWCNT-COOH induced ROS generation stained with dichlorofluorescin diacetate (DCFH-DA) in ARPE-19 cells.

ROS generation (Figure 3(c)) than the untreated MWCNTs (Figure 3(b)), indicating the useful modification of MWCNTs. Figures 2(d) and S5 showed the increasing ROS generation in percentage over 24, 48 and 72 h. Similarly, increased ROS generation was found to be time and dose dependent (Figure 2(d)). MWCNT-COOH induced the lowest ROS generation among these three types of MWCNTs while MWCNT-OH exhibited lower ROS generation than the untreated MWCNTs, confirming the plasma modification especially with carboxyl is an efficient way to improve the genotoxicity of MWCNTs. This can be attributed to presence of functional groups on nanotubes may be useful to decrease activity of the surface effect, giving rise to the decline of ROS generation levels.

Caspase-3 plays an important role in the process of RPE cells apoptosis since the activation cytosolic caspase-3 and chromatin condensation are both indication of apoptosis. Apoptotic studies were carried out using fluorescence microscopy by staining cells with Hochest 33258 and the activity of caspase-3 was determined by Caspase 3 Activity Assay Kit [28]. Apoptosis caused changes in morphology of ARPE-19 cells such as compaction, condensation and segregation of the nuclear chromatin. Apoptotic cells are normally condensed nucleus with a round shape and showed enhanced fluorescent signal (arrows in Figure 4). The apoptotic ratio of cells induced by MWCNT-COOH was found to be less than 1.5%, indicating well compatibility of MWCNT-COOH. Figure 4(e) showed the increased activity of caspase-3 of ARPE-19 cells after culturing with MWCNT-

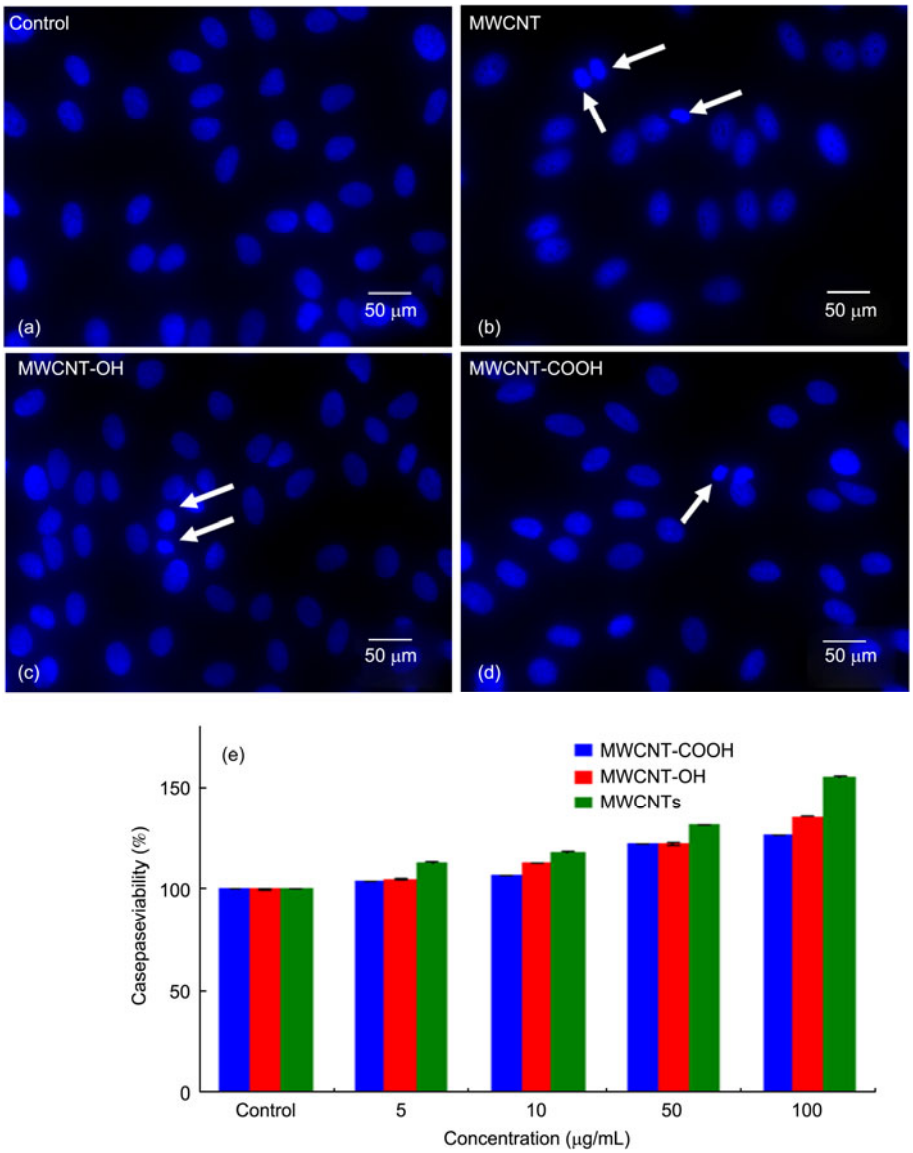

Figure 4 Fluorescence micrographs of Hoechst 33258 stained ARPE-19 cells incubated (a) without MWCNTs, (b) with MWCNTs, (c) with MWCNT-OH, (d) with MWCNT-COOH over 72 h. Arrows indicate cell apoptosis. And (e) percentage change in caspase generation after exposure of ARPE-19 cells to various concentrations of MWCNT, MWCNT-OH and MWCNT-COOH over 72 h.

based materials over 72 h. Similar with the previous studies in our work, more apoptotic cells were observed at the MWCNT containing samples when comparing with the other types. MWCNT-COOH induced lowest apoptosis cell among these three kinds of MWCNTs.

3 Conclusions

Our preliminary study demonstrated for the first time the ocular toxicity of MWCNTs and plasma functionalized MWCNTs (e.g. MWCNT-OH and MWCNT-COOH) with human RPE cells using various techniques, including optical micrograph, TEM, CCK-8 assay, LDH assay, ROS assay and Apoptosis assay. MWCNTs were found to be toxic to human RPE cells showing a decrease in cell viability, increase in LDH release, high percentage of ROS generation, and high content of apoptotic cells. Suitable personal protection is essential when dealing with MWCNTs. MWCNTs were able to be swallowed and released by cells without damaging cells which may be considered as a potential vector for ocular genetic diseases. Plasma modification, however, was found to be an efficient way to improve ocular biocompatibility of MWCNTs. Carboxyl and hydroxyl functionalized MWCNTs exhibited much better biocompatibility than the pristine MWCNTs. Particularly, MWCNT-COOH exhibited the best biocompatibility, providing efficient ways to improve toxicity of MWCNTs for practical applications.

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