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Carbon nanostructures as a scaffold for human embryonic stem cell differentiation toward photoreceptor precursors

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Carbon nanomaterials have been introduced as a scaffold for various biological applications due to their unique physical and electrical properties. Here we studied carbon nanotubes (CNTs) and carbon nanofibers (CNFs) as scaffold materials for the differentiation of human embryonic stem cells (hESCs) towards photoreceptor precursor cells (PRPs). We report on their cytotoxicity, their effect on cell morphology, cell-surface interface and the differentiation process. To this end, hESCs were differentiated into PRPs on carbon nanofibers (CNFs), long horizontal CNTs (LHCNTs), vertically aligned CNTs (VACNTs) or glass (control) surfaces. The differentiated cells were investigated by immunohistochemistry, fluorescence imaging and electron microscopy. Our results revealed that the investigated nanomaterials were not cytotoxic to the cells during the differentiation process. The surface interface effect on the cells was apparent, affecting cell directionality, migration and morphology. Interestingly, cell fate was not dependent on the substrate type, as inferred from the similar dynamics of the loss of pluripotency and the comparable expression levels of the photoreceptor marker Crx for all investigated substrates. These results are important for better understanding the effect of nanomaterial surface interaction with differentiating neural cells in general, and for future use of these materials as scaffolds for differentiating photoreceptors for vision restoration in particular.

Introduction

Cell therapy is an important emerging treatment modality currently being used for the treatment of a variety of disorders, among which are neurodegenerative diseases. Transplanted cells can treat or delay degeneration by cell replacement4 or alternatively by inducing neurotrophic effects that prevent or inhibit the death of the host tissue5. A main source of cells for this treatment modality is cells differentiated from human embryonic stem cells (hESCs) or induced pluripotent cells (iPSCs), both of which hold therapeutic promise due to their pluripotency3. Of great interest is the extensive research that has shown that light-sensitive photoreceptors differentiated from pluripotent cells can restore vision in blind patients suffering from degeneration of the photoreceptors4,5. Recent studies, however, have shown that only a small number of the cells will integrate with the host retina4,5.

Interestingly, studies have shown that biomaterials/scaffolds can serve as an effective tool for tissue engineering, in general6–10, and for enhancing and improving the survival and integration of transplanted photoreceptors cells, in particular11, probably by providing mechanical support, an organized structure and orientation12,13. Other studies have shown that various scaffold biomaterials can also have an effect on the hESC differentiation cell fate, both in vitro7,14 and in vivo15.

Owing to their unique mechanical and electrical properties, carbon nanotube (CNT) and carbon nanofiber (CNF) scaffolds have been widely used in biomedical fields for various applications such as biosensing16, tissue engineering17 and drug delivery18. Previous studies have reported on the biocompatibility of these carbon materials in vivo and in vitro19, with some studies demonstrating this biocompatibility on retinal cells20,21. Of particular interest are studies using CNT-based electrodes for subretinal implantation for vision restoration21,22; with results again highlighting the biocompatibility of such materials with retinal tissue23.

Notwithstanding the available reports, there is a lack of information regarding the toxic effect of CNTs on various cell lines, both in vitro and in vivo24,25 and their effect on the fate of differentiating neurons, and specifically photoreceptors, is not yet known.

Here we investigated, for the first time, to the best of our knowledge, the effect of various carbon-nano structures on differentiating cells, specifically hESCs differentiated towards photoreceptor precursors (PRPs).

Our results reveal that these substrates have no apparent cytotoxic effect on the differentiating cells. We also observed that these substrates reduce the extent of cell migration during differentiation, compared with a control substrate, and that the long horizontally aligned CNTs (LHCNTs) also affect migration directionality and cell morphology, thus potentially enhancing integration with the host.
retina. Interestingly, we found no significant effect of the various substrates on the differentiation toward PRP cells, compared to the control substrate. Taken together, these results indicate the potential of these nanostructures to serve as a scaffold for the differentiation of hESCs toward PRPs or other neuronal cells; they may form the basis of optimizing scaffolds for cellular therapy for vision restoration.

Results and Discussion

Synthesis and characterization of carbon nanostructures

To investigate the effect of various carbon structures, we synthesized three types of carbon nanostructures. The first type was 1.6 mm-long Vertically Aligned CNTs (VACNTs) (Fig. 1a, 1b), synthesized using atmospheric pressure chemical vapor deposition (CVD) 26,27. High-resolution transmission electron microscopy (HRTEM) analysis of the dispersed VACNTs (Fig. 1c) revealed that the CNTs have an average diameter of 7 nm with 4 walls and exhibit a high degree of crystallinity. The second type was Long Horizontally Aligned CNTs (LHCNTs) (Fig. 1d, which was flipped on its side). This dense structure was also grown using CVD but with different process parameters leading to much longer CNTs (4 mm-long 28) (Fig. 1e). The LHCNTs' diameter was found to be approximately 40 nm and with 20 walls (Fig. 1f). Finally, Carbon nanofiber mats (CNFs) (Fig. 1g-i) which were synthesized using a previously published method 29. Figure 1g and 1h show a photo image and a SEM image of CNFs, respectively. HRTEM (Fig. 1i) of the CNFs from the delamination-enabled mats shows the catalytic bidirectional growth of CNFs with embedded catalysts and diameters ranging from 70 to 160 nm, with an average of 105 nm and a standard deviation of 23 nm.

1. Vertically aligned CNTs (VACNT) => Cells on top
2. Long horizontal CNTs (LHCNTs) (sample flipped 180°) => Cells on the side
3. Carbon nanofiber mats (CNF) => Cells on the top

CNT and CNF functionalization for embryoid body (EB) seeding

The various substrates used in this study were found to be highly hydrophobic, in agreement with previous reports (Fig. Supp. 1), thus preventing effective cell growth and attachment. We therefore functionalized the surface by exposure to UV/ozone, which significantly decreased the surface hydrophobicity 29, as revealed by the decrease in the contact angle measured using the droplet method (Fig. Supp. 1). The control glass surface, in contrast, did not require any functionalization because the contact angle of the untreated glass was similar to that of an untreated nanostructure. Furthermore, although it has previously been reported that the UV/ozone treatment can affect the structure 30, herein SEM images (Fig. Supp. 2) revealed that surface functionalization did not result in any apparent structural changes in both the CNT and CNF surfaces (Fig. Supp. 2).

Our results are in agreement with results obtained when similar methods were applied for the functionalization of such hydrophobic substrates to allow for cell seeding 30,31. More importantly, UV/ozone surface treatment enhanced cell growth and adhesion (as will be shown next) with no additional surface coating required (e.g., Matrigel and laminin 32).

Cytotoxic effect of CNT and CNF as a substrate for hESC differentiation after 7 days

Embryoid bodies (EBs) were produced as described in detail in the experimental section. Next, the EBs were seeded on three types of nanostructure surfaces and the differentiation process was monitored for 24 days, as shown in the schematic illustration of the study design (Scheme. 1).

Scheme 1. Schematic illustration of the experimental paradigm for investigating hESC differentiation towards PRPs on the various carbon surfaces. GFP-positive hESCs co-cultured with feeder stromal cells (a) were seeded on a microwell dish (b), which resulted in the generation of uniformly sized embryoid bodies, at which point the differentiation process commenced (day 1). (c) At day 7, EBs were seeded on the various carbon surfaces (CNF, LHCNT and VACNT). (d) The differentiation was completed on day 24 and the differentiated cells were investigated for viability, morphology, directionality, migration, adhesion and differentiation.

To study a possible cytotoxic effect of the carbon scaffolds, the cell viability was examined by proTUNEL assay (staining for apoptotic cells) seven days following the seeding of the EBs (Fig. 2a-d). As a positive control, the staining protocol was applied on ethanol treated ARPE cells (Fig. Supp. 3). We found no significant reduction in the viability of cells cultured on the various nanostructures, compared with the control (EBs seeded on glass) (Fig. 2e) (p>0.05, Student’s t-test ± SD n=3).

In addition to the proTUNEL assay, the expression level of GFP protein, a reliable indicator of our cells viability 33, showed no decline.
after 7 days following culturing on the nanostructures, further suggesting the viability of the cells (Fig. 2a-d). The effect of carbon materials on cell viability is still unclear, with seemingly contradictory results being reported. Some studies showed toxic effects\cite{34,35}, whereas others did not\cite{36,37}. Thus, our results significantly contribute to this field by revealing that none of the investigated carbon nanostructures had an effect on cell viability during the differentiation process.

**Morphological changes**

We observed that the various nanostructure surfaces had a significant effect on the PRP morphology. It was very noticeable that there are significantly more extensions and protrusions in cells that were grown on the CNFs, compared with other surfaces (Fig. 3a-h). Furthermore, the cells on the LHCNTs were elongated, in contrast with the rounder and more symmetric cells grown on the other surfaces (Fig. 3c, 3c',3g). This was also evident by analysis of the nuclei geometry, with the nuclei of cells grown on LHCNTs showing a significantly higher aspect ratio (1.87±0.4), compared with the nuclei of cells grown on other substrates (1.59±0.4 control, 1.53±0.3 CNF, 1.58±0.2 VACNT), (P<0.05, Student’s unpaired t-test for all surfaces compared to the control. The scale bar is 100 µm.

![Fig. 2. Cytotoxic effect of CNT and CNF as substrates for hESC differentiation. After 7 days of differentiation, proTUNEL-BrdU staining (red) of apoptotic cells, showed no significant cytotoxic effect on cells cultured on all the investigated surfaces. (a) Glass control. (b) CNFs. (c) LHCNTs. (d) VACNTs. (e) The percentage of viable cells differentiated on the various substrates. Green: GFP, Blue: (nuclei), Red: proTUNEL-BrdU staining of apoptotic cells. p>0.05, Student’s unpaired t-test for all surfaces compared to the control. The scale bar is 100 µm.](image)

The morphology of the differentiated cells appeared to be different on the various surfaces, especially the elongation of the cells on the LHCNT surface. This result is in agreement with previous studies showing the effects of horizontal carbon nanotubes\cite{38,39} or other similar materials with a clear orientation\cite{40}. The morphological change may arise from the different topographical cues of the various substrates, as reported by others\cite{41,42}. It has been shown that topographical cues, such as nano-lines, can affect neuronal morphology and adhesion, and the rate of neurite formation\cite{41}. Others have shown that nanopillar arrays significantly increase the number of axon collateral branches and also promote their growth\cite{44}. Our study enabled us to study the effect of nanoscale topographical cues on the neuron cell body during development, showing a marked effect in the early stages of the neuron’s development, which, interestingly, extended from the cell body to the nucleus.
Fig. 3. The effect of the various substrates on cell morphology. (a) hESCs differentiated on a glass control substrate. (b) hESCs differentiated on CNFs (white arrows point to the protrusions emanating from the cell body). (c) hESCs differentiated on LHCNTs (white arrows point to the fiber’s directionality). (d) hESCs differentiated on VACNTs (the scale bar a-d is 100 µm). (a’-d’) An enlargement of the areas demarcated in a-d. (The scale bar in a’-d’ is 40 µm.) (e-f) Confocal imaging showing the morphology of GFP-hESC differentiated cells on the various substrates; (e) control, (f) CNF, (g) LHCNT, (h) VACNT. The scale bar e-h: 50 µm. (i-l) Nuclei staining of cells on the various substrates; the scale bar is 20 µm. (m) Quantification of the aspect ratio using the nuclei staining in i-l, defined as the ratio of the cell nuclei diameter in both directions, for the various substrates. *P<0.05, t-test.
Migration of cells differentiated on the various nanostructures

Cell differentiation during development in-vivo or in-vitro is highly dependent on the geometrical arrangement of the cells that affect the concentration gradient of many growth factors and cell-to-cell communication factors. Previous works have reported on how cell migration affects the fate of the differentiating cells and the effect of the micro-environment around the embryoid body (EB) on the differentiation process. We assumed that the nanostructure surface, and mainly the CNF, which possesses 3D surface characteristics, would affect the migration of cells far from the EBs. Indeed, we found that the migration area of cells cultured on the control (glass) was significantly higher compared with the other nanostructure surfaces, as shown in Figure 4e (P<0.005, n=5/group, average± SD).

Further quantification showed that cells differentiating on all nanostructure surfaces migrated to a smaller distance compared with the control, with the smallest distance found for CNF (Fig. 4f) (P<0.05, n=5/group, average± SD).

Our results show the significant surface effect on the migration distance and area compared to the control. In particular, the CNF exhibited a significantly low distance migration compared with the other carbon surfaces.

Previous reports have shown that the migration of differentiating cells away from the EBs and the distance covered during the differentiation process have a significant effect on cell fate. We therefore further studied the effect of the various surfaces on the differentiation fate of the cells, as is described below.

Growth directionality of differentiated cells on the various nanostructures

An additional investigated feature of differentiating stem cells is growth directionality, which is important for the differentiation of pluripotent cells. Since the nanostructure surfaces utilized in this study varied in their characteristics, we expected to observe a clear directionality of cells along a trajectory away from the EBs and that a greater effect would be observed on cells seeded on LHCNT, which is characterized by a clear directionality. Indeed, cells grown on LHCNT adopted a direction aligned with the nanotube directionality (Fig. 5 c,c’), compared with the other surfaces where no specific directionality was observed (Fig. 5).

A good measure to quantify cell directionality is nuclei angle, which is a good indicator of the cell’s directionality. This analysis revealed an angle distribution of 0-30 degrees for the majority of cells relative to the LHCNT orientation (Fig. 5g). In contrast, the quantification did not reveal any clear directionality for cells grown on the other surfaces (Fig. 5 e,f,h), where there was a more uniform and wide distribution of the nucleus angles.

This observation is of great importance, since the advantageous effect of cell directionality on obtaining the desired integration of transplanted cells with tissue has already been reported. Moreover, the orientation of transplanted photoreceptor cells by a scaffold is believed to increase the integration of these cells into the host retina. Future studies should evaluate the potential of using a carbonaceous scaffold for eliciting directionality in implants used for retinal replacement therapy.
Fig. 4. Quantification of the degree of migration of cells differentiated on the various nanostructures. Cell migration from EB (denoted by *) on (a) glass, (b) CNFs, (c) LHCNTs and (d) VACNTs. The characteristic dispersion area is denoted by arrows in (a). (e) Quantification of the cell migration area which was defined as the ratio between the cell dispersion area and the Eb area. (f) Quantification of the maximal cell migration distance away from the EB edge. (*) for both (e) and (f) - P<0.05, t-test. The scale bar is 400 µm.

Fig. 5. Migration direction of differentiated cells on the various nanostructures: (a) glass substrate, (b) CNF, (c) LHCNT and (d) VACNT. Green: cells GFP. * denotes the EB area at the various surfaces. Scale bar: 100 µm. (a’-d’) enlargement of the area demarcated in panels a-d. (the scale bar a’-d’ is 40 µm). (e-g) Nucleus angle distribution histogram for control, CNFs, LHCNTs and VACNTs, respectively.
Focal adhesion of the differentiating cells

In addition to studying the effect of the various surfaces on cell migration, we investigated the cell’s interaction with the nanostructure surfaces. In order to study the surfaces’ interaction, we acquired cross-sectional images of cells on the nanostructure surfaces using FIB/SEM techniques to visualise the cell-surface interface (Fig. 6). These cross sections of the various substrates revealed good cell-surface coupling, with close proximity between the cell membrane and the surface.

Quantification of Actin expression on the various substrates was defined as the ratio of the actin area to that of the total cell area (visualised by the GFP expression). This analysis revealed a higher expression of Actin on LHCNT and VACNT substrates, compared with CNF and control substrates (Fig. 7i).

Our results are in agreement with those of other studies reporting increased actin expression on carbon nanostructures, compared with a glass substrate.

In order to further study the cells’ interaction with the nanostructure surfaces, we investigated the presence of focal adhesion proteins, which play an important role in the interaction of cells with the surface. To this end, we stained for vinculin and actin, which are widely used focal adhesion and cytoskeleton markers. The cytoskeleton staining by Actin was robust and similar for cells grown on all surfaces (Fig. 7a-d). In addition, focal adhesion complexes (FACs) were clearly found for all types of surfaces, indicating good cellular adhesion on the various substrates (Fig. 7e-h).

The presence of the focal adhesion protein in cells differentiating on the various carbonic nanostructures, with good contact between the cells and the structure, is an important finding, since these proteins play a critical role in cellular differentiation and embryogenesis, as reported by several groups, and are also important for improving the electrical coupling between cells to electrodes composed of carbonic structures, in line with the pivotal studies by Hanein group and others.
Finally, previous reports have shown that focal adhesion proteins play an important role in differentiation processes; therefore, directly affecting the fate of the differentiating cell. Interestingly, these proteins also affect cell migration, which is again known to have an important effect on cell fate. We therefore studied the differentiation fate of cells cultured on the various surfaces toward PRP, as will be described next.

Effect of substrate on the differentiation of hESCs toward PRP

The effect of surface characteristics on the differentiation of hESCs toward various lineages in general, and towards neurons in particular, is of great interest. Despite some reports on the effect of the CNTs and CNFs on the hESC differentiation process into several types of cells such as adipose cells and neurons, many unknown factors remain to be studied in this field.

We therefore studied the effect of the nanostructure surfaces on the pluripotency, proliferation, and differentiation of hESCs toward PRP after 24 days of differentiation.

To study cell pluripotency, we utilized Oct-4 levels as a measure (Fig. 8a-f). We found that the various surfaces did not have a significant effect on the loss of pluripotency, as was evident by a similar, almost complete loss (P > 0.05) of Oct-4 expression for all surfaces compared to hESC (P < 0.05) (Fig. 8a-e middle panel, and Fig. 8f). Similar results were found for the proliferation marker Ki-67 (Fig. 8a-e lower panel and Fig. 8f). It can be inferred from these investigations that the various surface characteristics did not significantly affect the decrease in pluripotency and proliferation ability during the differentiation process, compared with a glass control surface.

Fig. 8. Substrate effect on the proliferation and pluripotency of the differentiated cells (a) hESCs stained for nuclei, OCT4 and Ki67, respectively. (b)-(e) Cells differentiated on glass substrate (control), CNFs, LHCNTs, and VACNTs, respectively. Blue: nuclei staining, red: OCT-4, a pluripotency marker, Magenta: Ki-67, a proliferation marker. (f) Quantification of the percentage of cells expressing each marker for the various substrates. Expression of Oct-4 was almost zero for all cells except for hESCs. The scale bar is 50 µm. *P < 0.05, ** P > 0.05, t-test.
To further evaluate the effect of the surfaces on the differentiation process, we investigated the percentage of cells stained for the photoreceptor precursor marker CRX\(^{68,69}\) in cells differentiating on the various nanostructure surfaces (Fig. 9a-d). Quantification of the expression levels indicated a yield of about 30%-40 CRX-positive cells on the various surfaces including the control surface (Fig. 9e). These results indicate differentiation of the hESC toward the desired direction of PRP cells on all surfaces. A comparison between the various surfaces found no difference in the differentiation yield toward PRP (P>0.05).

**Fig. 9.** Characterization of PRPs seeded on the various substrates using the PRP marker CRX (red) and nuclei staining (blue). (a) Cells differentiated on glass substrate (control), (b) CNFs, (c) LHCNTs (d) and VACNTs (e). Quantification of the percentage of cells expressing the Crx marker for the various substrates (scale bar for a-d - 50 µm).
These results reveal that there is good differentiation on all investigated surfaces, with stem cells losing their pluripotency and differentiating toward PRP. Interestingly, although our results are in agreement with some studies showing no adverse effects of carbonaceous surfaces on the differentiation of neurons, they are in contrast to others that have shown either a decrease or an increase in the differentiation of cells grown on the various substrates. It should be mentioned that in the current research we investigated the differentiation process at a single time point following a short differentiation process. Future studies should study a longer differentiation protocol for more mature photoreceptors and further address the kinetics of the differentiation at several time points.

In a very important paper, Johnen et al. reported that the CNT surface affects retinal precursors gene profiling and cell survival, which suggests the applicability of these surfaces for coating retinal implant electrodes. Our findings further support the use of these materials for either electrode surface modifications or as scaffolds for retinal stem cell implantations.

**Experimental**

**Synthesis of VACNTs**

VACNT synthesis was performed as previously reported. Briefly, VACNTs were synthesized in a three-zone atmospheric-pressure furnace, using a single fused-silica tube with an internal diameter of 22 mm. The incoming gases were preheated to 770 °C and flowed through the first two zones before reaching the sample, which was positioned in the third zone for annealing and growth steps (755 °C). The flow of He (99.9999%), Ar (a mixture of 99.999% Ar with 1% oxygen), C2H4 (99.999%) and H2 (99.9999%) (Gas Technologies) were maintained using electronic mass flow controllers (MKS model P4B) with a digital mass flow control unit (MKS model 247D). Flow rates of the annealing gas mixture of helium and hydrogen were 100 and 400 standard cm3/min, respectively. This gas mixture was flowed for 15 minutes while all furnaces were set to the desired temperature. Once the set temperatures of all furnaces were reached, the quartz tube was shifted, positioning the sample in the growth zone to start the annealing process (5 minutes). The annealing step was followed by the growth step. The flow of He, H2, Ar, O2 and C2H4 were respectively set at 100, 400, 100 and 200 sccm (standard cubic centimeters per minute), for 90 minutes. After the growth was completed, the quartz tube section with the sample was pushed out from the heated zone of the furnace to slowly cool down to room temperature under a flow of helium before removing the sample from the furnace.

Here we cultured the cells on top of the vertically aligned CNTs (termed VACNTs), and on the long axis of the CNTs by flipping the CNT carpet on its side (termed long horizontal CNTs (LHCNTs) (Fig. 1).

**Synthesis of CNFs**

CNF growth was carried out as previously reported. Briefly, we used an n-type Si (100) wafer coated by thin films of Ni (5 nm) over Pd (200 nm), with a Ti adhesion layer, all deposited using e-beam evaporation without breaking vacuum at a pressure of 1 X 10⁻⁶ Torr. Catalyst pretreatment, followed by CNF growth, was performed using the same furnace as mentioned before. The first two zones preheated the source gases (at 770 °C), while the sample was positioned in the third zone for the annealing and growth steps (650 °C). The annealing time was 30 seconds and the growth duration was 60 minutes. The cells were cultured on top of the CNFs.

**Carbon Nanostructures characterisation**

CNTs and CNFs were characterised using a field emission SEM (FESEM; FEI, Helios 600) operating at 5 keV. High-resolution transmission electron microscope (HRTEM) studies were performed using a JEOL-2100 field emission gun TEM operating at 200 keV equipped with an EDAX. Samples were prepared by dispersing a section of the CNT carpet and CNFs in isopropanol with gentle sonication for an hour, and then placing one drop of the dispersion on a 300 mesh Cu lacey carbon grid (from SPI).

**Functionalization of CNT and CNF arrays**

CNT and CNF arrays were oxidized with a UV/ozone cleaner (UVOCS Inc., RO BOX 543) for 10 min prior to use. The degree of wettability for the CNT and CNF arrays was analyzed using the water droplet method. The water droplet, 5 µL in volume, was used in the
measurements. The contact angle measurement was repeated four times and averaged for each sample.

Photoreceptor Precursor Generation

Photoreceptor precursors (PRPs) were generated as previously reported by our group. Briefly, human ES cells (hESCs, US National Stem Cell Bank (WA09)) were grown on mitomycin C inactivated STO cells (a murine line derived from embryonic fibroblasts in NutriStem® hPSC XF Culture Medium (Biological Industries, Israel, 05-100-1A). The medium was changed every other day. hESCs were trypsinised to single cells and seeded in differentiation medium in agarose microwell molds (9,000 cells per well) created by silicone micro-molds. One-day post-seeding on the microwells, the medium was replaced with differentiation medium: GMEM (Gibco, 11710035) supplemented with 20% knockout serum replacement (Gibco, 10828028), 0.1 mM non-essential amino acids (Biological Industries, Israel, 01-340-1B), 1mM pyruvate (Biological Industries, Israel, 03-042-1A), 0.1 mM 2-mercaptoethanol (Sigma, M7522), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin (Biological Industries, Israel, 03-033-1B). Next, 20 mM Y-27632 (TOCRIS, England, 1254) and 3 mM IWR1e (TOCRIS, 3532) were added to the medium up to day 12 and 0.1% Matrigel (GFR, BD Biosciences, FA354230) was added from day 2. On day 7, EBs were incubated separately on each of the nanostructure surfaces (LHACNT, VACNT and CNF). On day 12, 10% FCS was added. From day 15 to day 24 the medium was supplemented with 3 µM CHIR99021 (TOCRIS, 4423/10) and 100 nM SAG (TOCRIS, 4366).

Cell viability assays

Cell viability was evaluated on day 7 of the incubation using the proTUNEL DNA Fragmentation Assay (GTX855884, GeneTex), which stains for apoptotic cells. To this end, cells were fixated using 4% paraformaldehyde (Sigma, Israel) and washed with PBS. Next, the cells were stained with Br-dUTP (bromolated deoxyuridine triphosphate nucleotides) which is readily incorporated into DNA strand breaks, following the manufacturer’s instructions. The same cells were stained for Hoechst to visualise nuclei. Using confocal microscopy, cell viability was quantified by counting the percentage of dead cells out of the total number of all nuclei. As a positive control for apoptotic cells, ARPE cells were treated with 100% ethanol to induce apoptosis and then were stained using the same protocol. Supp Fig 3 shows the expected high percentage of apoptotic cells.

Immunocytochemistry

After 24 days of differentiation, the cells were rinsed in PBS and fixed with 4% paraformaldehyde (Sigma, Israel) for 25 min at room temperature and then rinsed again with PBS with 0.5% Triton-x 100 and 1% Tween (PBST). Blocking was performed for 30 min in 1% bovine serum albumin in PBST. Following an overnight incubation at 4°C with anti-human CRX antibody (mouse, ABNOVA, 1:100), Anti-Vinculin antibody [EPR8185] (ab129002), Anti Ki67 (AB15580 abcam) and Anti OCT-3/4 (Santa Cruz Biotechnology, Inc., (C-10): sc-5279), cells were rinsed with PBST, and secondary antibodies coupled to Alexa 594 (Jackson ImmunoResearch, 715-585-150) and 634 (Jackson ImmunoResearch, 715-585-150) were applied for 60 min at room temperature. Cells were rinsed in PBS. Hoechst 33258 (Sigma, B2883) was used for nuclear counterstaining. Actin staining was performed using Phalloidin (Cat. # PHDN1-A).

Morphological, orientation and migration distance measurements

To assess the morphological changes in the differentiated cells seeded on the various nanostructure substrates, the cells nuclei were imaged and analysed using ImageJ (Feret’s diameter parameter). Cell elongation was investigated based on the aspect ratio (major axis/minor axis) of the cells from three random captured images (n =200 cells per group, in triplicate). Furthermore, the orientation angle was calculated based on the angle of the cells relative to the base line (the horizontal line for the glass, LHACNT and CNF substrates and a line parallel to the CNT directional for the VACNT substrate; n =100 cells per group, in triplicate). Distance and area migration were measured using imageJ on the images acquired by a Leica Stereoscope (Leica dfc7000 T) and analysed using ImageJ (area and distance). The cell migration area was defined as the ratio between the cell-dispersed area normalised by the EB area.

Cultured cells were visualised using a confocal microscope (Leica TCS SP8). No features were removed or added digitally.

Scanning electron microscopy (SEM) and focused ion beam (FIB) imaging

Samples were processed similarly to our previous report. Briefly, samples were washed using PBS at 37°C (Biological Industries, 02-023-1A) 2 times (5 minutes each) and were primarily fixed with Karnovsky fixative buffer consisting of 2.5% (wt/vol) paraformaldehyde, 2.5% glutaraldehyde and 0.1M cacodylate buffer (EMC, 15949) for 1h at room temperature and then left overnight at 4°C. Next, the fixative was washed three times with 0.1M cacodylate buffer, and samples were further processed by the CPD drying method using a Leica automatic EM CPD300 for 32 runs, 301 with a cooling temperature of 7ºC, a heating temperature of 40°C, the stirrer speed set to 302 100% and 100% advanced slow gas-out. The dried samples were then coated with a 20nm gold layer (Quorum Q150T ES) and imaged by E-SEM 326 (Quanta FEG 250 by FEI). Focused ion beam cross-sectioning was performed with a Helios 600 (FEI company) at the Bar-Ilan Institute for Nanotechnology and Advance Materials (BINA).
Conclusions

In this work we have presented a detailed investigation of the effect of three different surfaces of carbon nanotubes and nanofibers (namely, LHON Ts, VAON Ts and CNFs) on the differentiation process of embryonic stem cells toward photoreceptor and precursor cells while studying the viability, morphology, migration, adhesion, proliferation, pluripotency and differentiation efficiency.

The obtained results revealed that although there was a clear effect of the surface interface on the cells, as was evident by the pronounced effect on the morphology and the migration of the differentiating cells, there was little to no effect on the viability, pluripotency and apparent cell fate.

These results can contribute to the existing knowledge on retinal cell interaction with carbon nanotubes and can aid in determining the optimal electrodes’ surface modification materials or scaffold to be used in neural stem cell-based therapies in general or in the field of vision restoration, in particular.

Conflicts of interest

There are no conflicts to declare.

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