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Does Geometry Matter? Carbon Nanotube-Based Films, Ribbons and Fibers as a Platform for Neuronal Cells Culturing †

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Abstract: Carbon nanotubes (CNTs) became a fascinating nanomaterial for biomedical field where non-soluble forms also called "assemblies" have found the great eventual use. Because of the great electrical properties, assemblies of CNTs are a perspective material for electrical scaffolds manufacturing and electrodes fabrication. Electrically conductive materials with biocompatible properties are in the great importance for neuronal tissue engineering, registration of evoked potentials and electrical signals transmission.

Despite the numerous studies, there is still a big gap in realizing of these scaffolds' geometry and surface impact on cells physiology. Besides, only small portion of works describe assemblies of CNTs as scaffolds, the majority is concentrated on the composites that does not allow to highlight CNTs impact. Thus, in our work we address the issue and study regular films made of CNTs through standard chemical vapor deposition technique, flat in shape ribbons and twisted 3D cylindrical fibers for their effects on cells viability and morphology.

Firstly, to find if there any release of CNTs films components negatively affecting cells, we incubated Neuro2A cells with CNTs films floating in the media. After 1 and 7 days of incubation with films, by application of standard Alamar Blue test we revealed that Neuro2A cells have the same number of alive cells in the control (95±7%) and in the experimental group (100±10%). Standard LDH-assay allowed us to compare amounts of dead cells; we found that number of dead cells is comparable for the control group (27±5%) and for the experimental cells (18±5%). This result mean that CNTs in the form of assemblies do not release some components in the media.

At the next step, we prepared substrates with three different geometries, attached to the bottom of petri dishes with PDMS polymer – films, ribbons and fibers. After 1 day, cells were actively adhered at the scaffolds made of fibers and films. Comparing with them, ribbons showed much worse adhesion of cells because of the inhomogeneous height revealed by profilometry, so the cells roll off to the lateral sides of ribbons. One of the most essential results is the observation of Neuro2A cells directly attached to a fiber characterizing by a 3D cylindrical shape. Calcein staining had shown that the cells morphology is comparable with the control group and cells after incubation onto the scaffolds were alive.

Summing up, the present work demonstrates the absolute safety of non-soluble forms of CNTs for in vitro models and shows cells adhesion on platforms based on CNT assemblies with different geometries that opens new perspectives of the material application in biomedicine.

Keywords: carbon nanotubes, biocompatibility, nanomaterials, neuronal cells.

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1. Introduction

Carbon nanotubes (CNTs) are a well-known nanomaterial with diverse applications in electronics [1], composites manufacturing [2], water purification [3], and biomedical fields [4]. Starting from 1991, when a well resolved evidence of CNT has been presented [5], a vast number of studies appeared that were devoted to the extensive research of their mechanical [6], electrical [7] and optical properties [8]. These studies formed the foundation for the continual incremental research devoted to material and system property enhancement, often seen in micro material manufacturing. As a result, materials such as artificial muscles [9] and synthetic high-performance fibers [10] have been fabricated, breaking the barrier for nanomaterial application at the industrial scale. In addition, synthesis techniques have undergone major advances that facilitated a prominent increase of CNT annual production, currently reaching several hundreds of tons for some companies raising a question about CNTs safety [11–13].

Nowadays, there are many ways of CNT assemblies' manufacturing [14] depending on factors such as variations in geometry starting from thin flat films, ribbons, and ending up with cylindrical fibers with helical [15] and more advanced shapes [16]. Because of the electrical performance and flexibility shown by such structures, they are ideal scaffold candidates for electrically active cells such as muscle and neuronal cells [17–20]. The diversity of CNT assembly geometries opens a broad avenue on the road toward application of the material for peripheral nerves restoration [21,22], electrically conductive scaffolds manufacturing and use in 3D biological model reconstruction [23,24], as well as for a complete shift from metal electrodes to CNT-based electrodes for deep brain stimulation [25,26]. In addition, recent studies showed that CNT-based electrodes could be used for biosensing [27] of molecules such as glucose, dopamine, adrenaline, *etc.* in real time. When in contact with live cells, CNT scaffolds may be applied as a recorder of cell electrical activity or as a stimulator initiating cell firing and action potential generation. Compared to standard metallic electrodes usually applied for these tasks, flexible assemblies of CNTs are more biocompatible and stable in physiological media [13,18,28,29]. In such conditions, metals may actively degrade, corrode and release ions considered "aggressive" for such environments [30].

Initially, CNT films were tested in contact with neuronal cells [19,31] where electrically conductive CNT-based film was applied as a stimulator for cell action potential generation and cell morphological modulation [32] through the variation of substrates conductivity and thickness [33,34]. The first attempts had resulted in the fast development of the field, so today researchers are capable of installing real CNT-based electrodes in animal brains [25,35] or spinal cords for the imitation of injuries and repairing processes.

The precedence laid by previous research articles leads to the conclusion that CNT assemblies meet all the requirements for applications where contact with electrically active cells is desired for a stimuli generation and electrical activity recording. In addition, their properties are seen to be well suited for biosensing of a variety of biological molecules associated with diseases like Parkinson's disease, epilepsy, tremor, migraine, chronic pain, peripheral nerves damage, *etc.* [36,37]. CNT assemblies with different configuration may be applied in different research scenario. Thus, films are very useful in terms of the electrical stimulation of cells located on a flat surface, while ribbons and fibers could be applied as electrodes for animal studies or electrical scaffolds for 3D cell cultures. To the best of our knowledge, there has yet to be a study where the influence and impact of CNT assembly configuration on cell physiology is evaluated. Thus, our work is focused on searching for the impact of films, ribbons and fibers on cells adhesion properties, viability and morphology. The present study demonstrates resultswhich may be used for the construction of scaffolds and electrodes designing for wearable and implantable electronics. In addition, we present here a new convenient way of ribbons made of CNTs manufacturing for a laboratory scale production.

2. Materials and Methods

2.1. Carbon nanotube synthesis

Carbon nanotubes were synthesized using aerosol CVD method as described in the previous publications [14]. The synthesized tubes predominantly have an average diameter of 2.1 nm and a length of 20-40 μm [38].

2.2. Ribbons and Fibers manufacturing

Carbon nanotubes were collected on a nitrocellulose filter during CVD synthesis. For ribbons and fibers fabrication, the thin film made of CNTs was transferred to a glass slide and manufactured under the wet pulling protocol [39].

2.3. Cell culturing

Neuro2A, mouse neuroblasts, were purchased from ATCC and were cultured in standard DMEM (Cat. # 1.3.6.3, Biolot, Russia) and supplemented with 10% FBS (Cat. # 1.4.1.5, Biolot, Russia), L-glutamine (Cat. # 1.3.8.4, Biolot, Russia), 100 μg/mL penicillin/streptomycin (Cat. # 1.3.18, Biolot, Russia) at T25 flasks (Cat. # 707001, Nest, China). The media was replaced every 2-3 days and the cells were maintained in an incubator (5% CO_2 and 37 °C) (Innova CO-170, New Brunswick Scientific, U.S.A). For cells reseeding, we applied 1ml trypsin solution (Cat. # 1.2.2.6, Biolot, Russia) for 5 minutes for cells detachment from substrates. For cells counting, we applied Trypan Blue (1450021, Bio-Rad, Russia) and TC20 automated cell counter (Bio-Rad, U. S. A.).

2.4. Cell viability/cell mortality tests

Standard 96-well cell culture plates (Cat. # 0030128648, Eppendorf, Germany) were used for the viability and mortality tests. Cells were cultured with a density of 10^4/well in the culture medium and incubated at 37 °C under 5 % CO_2 during 24 hours before the contact with materials. After 24 hours, films of CNTs with a size of 5×5 mm were placed in the culture medium inside the experimental wells. Incubation time with the material was 1 day and 7 days.

After incubation with the material, cell viability was assessed through a standard test with a coloring agent Alamar Blue (Cat. # DAL1025, ThermoFisher Scientific, U.S.A.). In each experimental well, 10 μL of the fluorescent dye Alamar Blue was added and the fluorescence (540/590 nm) was registered with a spectrophotometer (Infinite F200 PRO, Switzerland). As a control, we used cells incubated in the same conditions without any material.

For the assessment of cell mortality, the standard LDH-assay (CyQUANT LDH Cytotoxicity Assay, Cat. # C20300, ThermoFisher Scientific, U.S.A.) was applied. For that, one half of the media from the experimental wells was transferred to new wells and a reaction mixture was applied. After addition of the reaction mixture and incubation for 30 minutes at 37 °C, absorbance was measured at 450 nm by a spectrophotometer (Infinite F200 PRO, Switzerland). For a control group, we used cells after lysing, *i.e.*, where all cells are dead.

2.5. Fluorescent microscopy

Films, ribbons and fibers were attached to the surface of petri dishes (Cat. # 30702115, Eppendorf, Germany) by polydimethylsiloxane (PDMS) (Cat. # 9016-00-6, SigmaAldrich, U.S.A.). After polymerization completion at 4 hours, the substrate was washed three times by PBS (Cat. # 1.2.4.5, Biolot, Russia). For substrate sterilization, we incubated them in 70% Ethanol (Bryntsalov-A Company, Russia) for 30 minutes. Cells were seeded in a concentration of 3×10^3/ cm^2.

Cell morphology visualization was done using the confocal imaging system Operetta (PerkinElmer, U.S.A.). Calcein (ThermoFisher Scientific, Cat. # C3100MP, U.S.A.) staining was used for identification of alive cells through incubation with the coloring agent for 10 minutes.

3. Results and Discussion

3.1. CNT films biocompatibility

To obtain information about the toxic effects for cells, we determined cell viability and mortality using Alamar Blue and LHD-test, respectively, after 1 and 7 days of co-incubation of cells with the samples. Figure 1, A, B shows the results of these tests where it is clearly visible that data for the test remain the one for the control groups.

CNT film was added to cells and incubated with them in order to reveal if there is some release of constituents from the film which may negatively affect the cells. The significant difference in cell mortality was shown only after 7 days, where the control group had a higher percent of dead cells (28%) when compared to the experimental group (19%). This fact could be explained by the differences in the speed of life cycle which was higher for the control group. Thus, the experiment showed the complete safety of CNT films through the acute toxicity test.

3.2. CNT films, ribbons and fibers as substrates for cell growth

Calcein Am staining revealed that all cells adhered onto the surface of the tested materials were alive (Figure 1, C). For film, we found that cells are more or less homogeneously distributed over the samples' surface, while for fibers we detected small agglomerates localized near the fibers surface. For ribbons, we found that cells are not actively adhered at the surface, but at the boarders of the material only. We propose that this is the result of the distinct geometry and surface stiffness, i.e., the films fully covering the bottom of petri dishes are flat and possess uniform thickness, while ribbons showed only 2-3 mm width with a height peak right in the middle of the sample, causing cells to roll off to the lateral lower portions. Among the tested geometries, only the fiber samples displayed a 3D shape, allowing the cells to form agglomerates with better adhesion around the material surface.

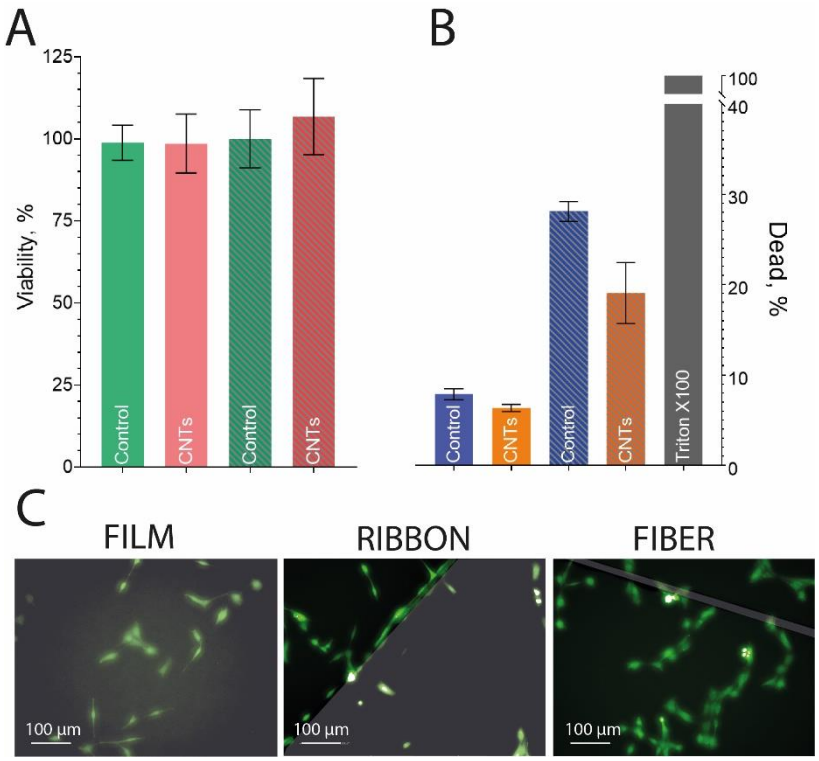

Figure 1. (A)Viability of the cell incubated with thin-films during 1 day, 7 days; (B) Cell mortality evaluated for day 1 and 7 days of incubation with CNT thin film.; Calcein staining (C) for cells after 1 day incubation with material; material is showed with a grey color.

4. Conclusions

The present study revealed that CNTs in the form of assemblies are completely biocompatible and absolutely safe for cells. After 1 days and 7 days incubation, data for viability and mortality for the experimental groups with materials are similar to the control cells grown on a regular substrate. Through fluorescence microscopy, we found that for fibers and films after 1 day of incubation, cells very actively adhered and spread at the scaffolds. For the ribbons, cells adhered rarely. Thus, the present work demonstrates neuronal cells growth on scaffolds made of CNT assemblies with different geometries.

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