

Basic Research for Peripheral Neural Regeneration by CNTs, A Preliminary “*in vitro*” Study

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Abstract

Purpose: The purpose of our study is to propose new insights into interactions between carbon nanotubes and neuron and glial cells electro-chemical activity.

Materials and methods: On each of N° 7 quartz substrates samples, N° 14 carbon nanotubes Spots have been prepared, grown on N° 7 Spots displayed in circular geometry and N° 7 on the base of different designed patterns and all vertically aligned. Every CNTs Spot were matched directly with groups of neuron and glial cells NOBEC derived from primary cells dissociated from neonatal rat olfactory bulb. In total N° 49 Spots in circular geometry and N° 49 in different designed patterns. Globally N° 98 Spots were tested, 14 of which were excluded for technical defects. The Study includes globally N° 84 Spots of CNTs.

Results: Scanning electron microscope observations showed that cells grew on CNTs and displayed numerous lamellopodes with signs of focal adhesion close to the CNT surface. From “*in vitro*” experiments we also observed: absence of cytotoxicity; cellular and molecular interaction with enhanced cellular proliferation; cellular axonal and dendritic regeneration; electrical stimulation and cells attraction towards CNTs assemblage. Additionally we observed the dissociative ability of glial and neuronal cells when matched and excited to upset the carbon nanotubes arrays.

Conclusion: Our study investigated the interactions between living system and nano materials at molecular level to check the ability of CNTs to enhance numerous cellular mythosis and significant activity and development of filipodia and lamellopodia structures as it happens at the new axons sprouts.

Keywords: Neural regeneration; Carbon nanotubes; Scanning electron microscope; Electrical stimulation; Tissue engineering; Nanofibres; Gastrointestinal absorption

Introduction

The purpose of our study is to propose new insights into the direct and specific interactions between Carbon nanotubes (CNTs) neuron and glial cells electro-chemical activity in order to improve our knowledge on basic and clinical regenerative nerve research and on tissue engineering. Recently in the field of neuroscience the application of single-walled (SWCNTs) or multi-walled (MWCNT) Carbon nanotubes has changed the approach to nerve-related research. There has been great interest in the biological and pharmacological use of nanomaterial's such as CNTs at the molecular and cellular scale such as nanotubes (gold, carbon etc), nanofibres, liposomes, nanoparticles, polymeric micelles, nano gels and dendrimers. MWCNTs have a simple and inert molecular structure, cylindrically shaped, of different size, and made by one or multi-sheets of graphene rolled up to form hollow cylinders (Figures 1 and 2). They constitute a flexible and enduring tool which, owing to high electrical conductance, is able to interface through its electrochemical properties with different organic molecules. Considering their diameters are similar to axons and dendrites, carbon nanotubes can also be utilized for biological and medical applications in basic and applied neuroscience. The interaction between CNTs and animal cells has been largely studied and their biocompatibility was widely ascertained utilizing CNTs as a substrate for neuronal growth [1-3] now this property is known to be very significant for future clinical applications. On the other hand, the assessment of the interaction of nanoparticles with biological systems *in vitro* as well as *in vivo* experiments has demonstrated the potentiality of health risk (inflammatory response) through inhalation, dermal

contact, gastrointestinal absorption and environmental contamination [4] Nevertheless in order to control health risk for humans, the hypothesis for a possible oxidative and cytotoxic potential of single or multi-walled carbon nanotubes led Yuan J [5] to study and ensure the absence of toxicity and innocuity in producing and processing CNTs if particular and strict cautions are observed during industrial processes or experimental manipulations. Today, the innovative neuroscience,

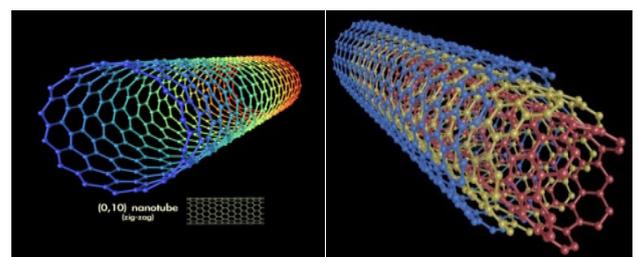


Figure 1: 3D models of single walled and multi wall edcarbon nanotube.

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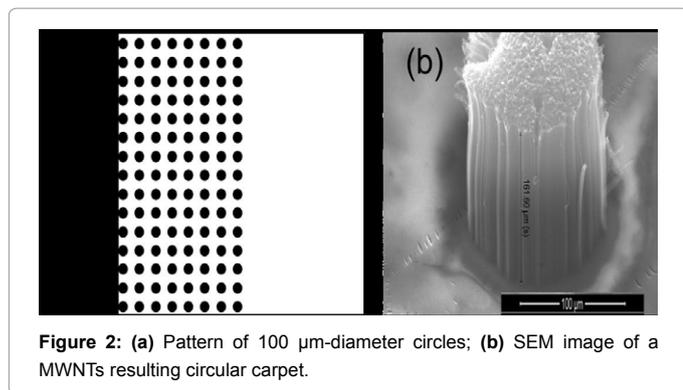


Figure 2: (a) Pattern of 100 μm-diameter circles; (b) SEM image of a MWNTs resulting circular carpet.

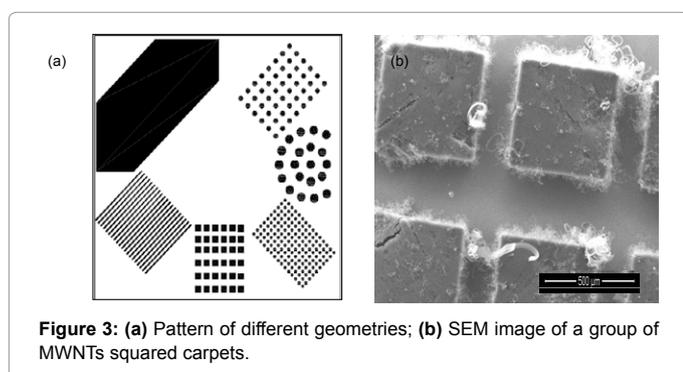


Figure 3: (a) Pattern of different geometries; (b) SEM image of a group of MWNTs squared carpets.

through CNTs physical and chemical features, may enhance regenerative strategies by means of intrinsic capacity to guide at the nano dimensional scale the specific interactions between synthetic materials and biological cell membranes [6]. Furthermore, in Xiang N and Wang G [7] researches ascertained that CNTs through their electrical conductivity produced enhanced migration and proliferation of Schwann cells, neuritis growth and axons regeneration, but in our opinion the ways in which they affect cellular function are still poorly understood. From 2010 our "in vitro" researches on the application of CNTs was focused on peripheral nervous system to study and control cells biocompatibility and regeneration in order to program a suitable axon extension after a peripheral nerve injury which produces a degenerative cellular and molecular disaggregation at the lesion site. We report here our experience and results on application of carbon nanotube technology to neuroscience and peripheral nerve research.

Materials and Methods

CNTs growth

A series of Carbon Nano Tubes (CNTs) samples have been prepared, grown on quartz substrates (2 cm × 2 cm of surface) on the base of different designed patterns (Figures 3 and 4). This research includes a study of N°3 patterns, each one containing 16 regularly-spaced spots of CNTs on quartz substrates, displayed in circular geometry and vertically aligned. Every spot was matched with groups of neuron and glial cells, in total 48 experimental items. A second, exactly similar study was performed through N° 3 patterns but with different spot geometries (in total all the same 48 experimental items). Globally 96 CNTs spots matched with neuron and glial cells. Exclusion: a third identical study was excluded for technical defects of CNTs grown on quartz substrates. Patterns were previously cleaned in acetone and patterned by photolithography micro fabrication procedure with 5 nm

thin film of iron catalyst, deposited by sputtering system. Multiwalled-Type Carbon Nanotubes (MWNTs) were used, each one constituted by 10 walls, vertically aligned with respect to the substrate surface, with lengths between 100 and 200 μm, and external diameters between 10 and 20 nm. Samples were prepared by using Low Pressure Chemical Vapor Deposition (LPCVD) technique in a quartz tube furnace (2 inch diameter) using ethylene as the carbon source gas at a 530 sc cm flow mass rate, 200 Torr pressure and temperature of 650°C, after the quartz substrates (20 mm × 20 mm of surface) were covered by a Fe film layer (5 nm), used as a catalyst, following the designed patterns (Figures 3 and 4).

Cell culture

The NOBEC cell line was derived from primary cells dissociated from neonatal rat olfactory bulb and immortalized by retroviral transduction of SV40 large T antigen [8]. NOBECs were grown for three days on spotted carbon nanotubes grown on quartz coverslips at 37°C in a humidified atmosphere of 5% CO₂/air, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 10% heat-inactivated Fetal Bovine Serum (FBS). Samples were fixed and processed for immunocytochemistry and Scanning Electron Microscopy.

Immunocytochemistry

After 3 days of incubation the samples were observed directly in the inverted biological microscope and also using confocal microscope after immunofluorescence reaction. For immunofluorescence, the samples were rinsed in PBS, blocked with normal serum (1%), (the use of a normal serum made in the same species of the secondary antibody is recommended), for 1 hr and then incubated overnight with the primary antibody. The samples were incubated with primary antibody against Ki-67 (monoclonal, mouse, 1:100, Novo Castra, Louis) that specifically recognizes proliferating cells. After primary antibody incubation, samples were washed three times in PBS and incubated for 1 h in a solution containing the secondary antibody (ALEXA 488 a-mouse) conjugated with a fluorophore and selected in order to recognize the specie of primary antibody. After three washes in PBS, samples were finally mounted with a Dako fluorescent mounting medium and stored at 4°C before being analyzed by a LSM 510 confocal laser microscopy system.

Scanning electron microscopy

SEM analysis was performed after fixation with glutaraldehyde (2.5%), washing in PBS and dehydration in ethanol (from 50% to

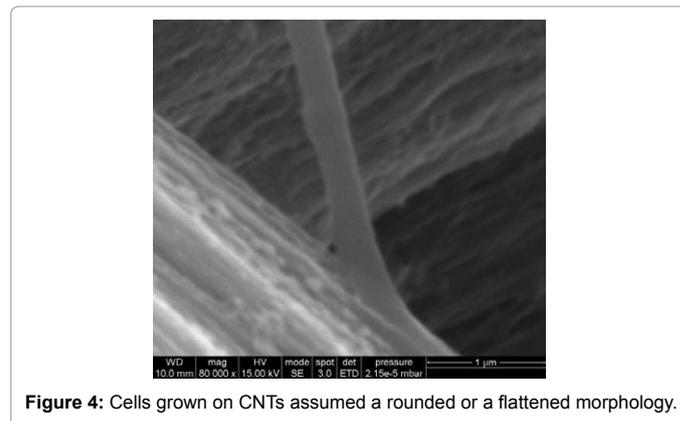


Figure 4: Cells grown on CNTs assumed a rounded or a flattened morphology.

100%). The MWNTs morphology was investigated using a Scanning Electron Microscope (SEM, Philips, ESEM XL 30) and a Transmission Electron Microscope (TEM, JEOL, Jem 2100 LaB6) (Figure 5).

Results

In vitro analysis was performed culturing glial cells (NOBEC) on quartz coverslip where CNTs were grown in regularly-spaced spots. Vitality of NOBEC cells was maintained over all the observation period (3 days) and normal proliferation rate of NOBEC cells and any sign of cell death were observed. Scanning electron microscope observations showed that cells adhered in a uniformed way on the support and grow also on CNTs. Cells grown on CNTs assumed a rounded or a flattened morphology. Glial cells displayed numerous lamellopodes with signs of focal adhesion close to the CNT surface (Figure 4). Then from "in vitro" experiments we overall observed that glial and neuronal cells matched with CNTs showed some significant and intriguing behavior as: Absence of cytotoxicity; cellular and molecular interaction with enhanced cellular proliferation; cellular axonal and dendritic regeneration; electrical or chemical stimulation and cells attraction towards CNTs assemblage. Finally we also observed the dissociative ability of glial and neuronal cells when matched and excited to upset the carbon nanotubes arrays (Figures 6 and 7).

Discussion and Conclusion

Our research investigated the interactions between living system and nanomaterials at molecular level to check the ability of CNTs to enhance cellular and molecular interactions on glial and neuronal performance. From our *in vitro* observations we think that specific interactions between nerve cells with CNTs, at the lesion site, might play an important role, in view of biological cell and membrane repair of the damaged axons, to obtain immediate reconnection with its target organs. We have exploited material properties of CNTs as

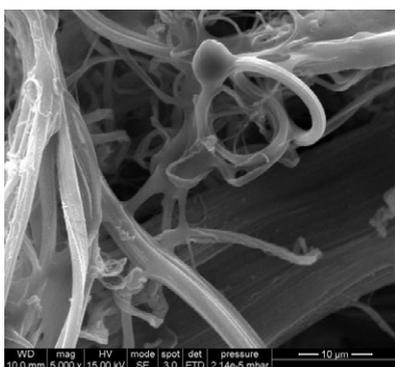


Figure 5: Glial cells displayed numerous filipodes and lamellopodes (Scanning electron microscopy).

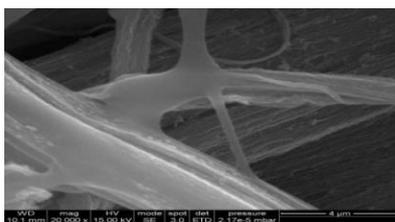


Figure 6: Glial cells displayed numerous filipodes and lamellopodes with signs of focal adhesion close to the CNT surface (SEM).

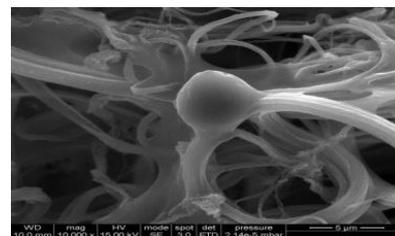


Figure 7: (SEM) Glial cells displayed numerous lamellopodes and filipodes.

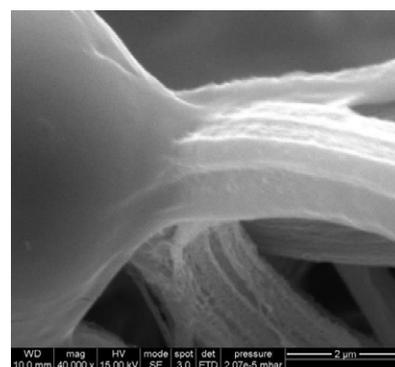


Figure 8: (Scanning electron microscopy) lamellopodes with focal adhesion close to the CNT surface.

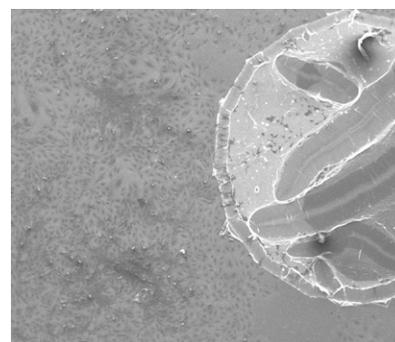


Figure 9: A SEM image of one of the circular carpet of CNTs matched to glial and neuronal cells; cells attraction towards CNTs assemblage. Additionally it can be observed the dissociative ability of glial and neuronal cells, when matched and excited, to upset the carbon nanotubes arrays.

strength, flexibility and electrical conductivity to sustain and promote neuronal electrical activity in networks of cultured cells as well as to deliver electrical stimulation to cells in contact with them. This specific biomolecular interaction not only promote cells' adhesion, due to a new probable presence of receptors and molecules of cell adhesion (important for axon growth and guidance), migration (probable presence of microspikes or dense actin meshwork), and proliferation (presence of numerous nuclear karyokinesis), but also mimic the function of conductivity of neural myelin enhancing cellular dendrite and neurite growth (Figure 6). Is this behavior consequent to high electric-physiological property to promote cellular and molecular interaction? or to chemical action on the neuron through membrane polarization and depolarization by Ca^{++} , necessary for a promotion of the neurite out growths? In conclusion, CNTs biocompatibility

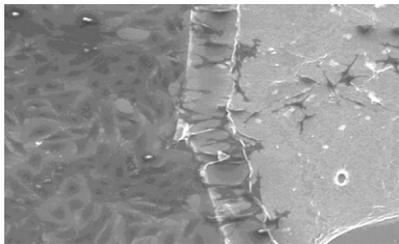


Figure 10: Further magnification of part of the carpet edge. Study confirms the absence of cytotoxicity of CNTs; the specific interactions between synthetic materials and biological cell membranes change the approach to nerve-related research. Electrical or chemical stimulation of CNTs enhances cellular proliferation and produces cellular axonal and dendritic regeneration.

is very significant for *in vitro* research as well as for future clinical applications but the ways in which carbon nanotubes affect cellular function are still poorly understood. The assessment of positive results in our *in vitro* research showed that glial and neuronal cells were really excited and activated; cellular and nuclear organs as mitochondria and endoplasmatic reticulum were reorganized in the cytoplasmatic area together with numerous cellular mythosis and significant activity and development of filipodia and lamellopodia structures at the new axons sprouts: this morphological change belongs to all clinical an injured peripheral nerve. Due to biocompatibility [9] and axonal growth promoting properties, these positive results authorize us to continue to apply this methodology also *in vivo* research for functional regenerative neuron activity (Figures 6 and 7), essential for future peripheral nerve

repair, neuronal tissue engineering and clinical outlook (Figures 8-10).

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