MORPHOLOGICAL ANALYSIS OF CORTICAL NEURONS CULTURED ON CARBON NANOTUBES AND NANOFIBERS

By

VALENTINA RAPAZZETTI

A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2013
To my great grandma, Natalina
ACKNOWLEDGMENTS

I thank my advisor Dr. Wheeler for his constant support and precious advice and Eric F. for his willingness, helpfulness and kindness at every stage of my research and without which this work would have not been possible.

I thank Dr. Yoon and his student PitFee J. from the Electrical and Computer Engineering Department for having kindly provided some of the samples used in this study.

I thank Dr. Baselli, Dr. Van Oostrom, Dr. Tran-Son-Tay and all the other collaborators of the ATLANTIS CRISP for giving me the opportunity to take part in such a prestigious program that is likely to be one of the most important experiences of my life.

I thank my lab mates Pan, Sanka and Dr. DeMarse for their enjoyable company, availability and their openness in discussing either scientific or just daily life topics.

I thank my parents Monica and Moreno, my grandparents Ivana, Giuseppe, Adriana and Antonio and my aunts, my uncles and my cousins for their spiritual and financial support.

Thanks to my beloved siblings Sarah and Lorenzo for their unconditional love.

Thanks to Margo for our immediate close friendship, for her reliability and generosity that I will never forget.

Thanks to Ana for been my always-smiling guardian angel during every single bad period I faced this year.

Thanks to Susi for been such a perfect flat mate, confidant and English referent.

Thanks to Ally, my favorite American in the universe, for been a supportive and loyal friend and for all the laughs we shared.
Thanks to Subho for his constant support during the final and hardest part of this work, for his accurate advice and his sensitivity, nonetheless for the delicious Indian food his mum made.

A special thanks goes to my “little Italy in Gainesville”: Elisa, Giacomo, Ilaria, Irene, Cosimo, Paolo, Agnese, Alessandro and especially Massimiliano for their warm welcome and the nice time we spent together.

Finally I want to thank my far-away but never so close friends Bela, Mari, Vale, Simone, Matteo, Fabio, Tommaso, Glory, Ciomba, “the Belfastian family”, Costi, Roberto, Rossella and Francy because my life would have been much harder and difficult without them.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>8</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>11</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>13</td>
</tr>
<tr>
<td>2 BACKGROUND</td>
<td>16</td>
</tr>
<tr>
<td>Neuronal Cell Growth and Surface Modifications</td>
<td>16</td>
</tr>
<tr>
<td>Substrates for Neural Cell Culture</td>
<td>17</td>
</tr>
<tr>
<td>Coating Materials for Neural Cell Culture</td>
<td>18</td>
</tr>
<tr>
<td>Studies on Surface Modifications and Related Neurite Growth</td>
<td>20</td>
</tr>
<tr>
<td>Carbon Nanotubes</td>
<td>25</td>
</tr>
<tr>
<td>Structure and Manufacturing Procedures</td>
<td>25</td>
</tr>
<tr>
<td>Properties and Applications</td>
<td>29</td>
</tr>
<tr>
<td>Functionalization Procedures and Applications</td>
<td>31</td>
</tr>
<tr>
<td>Carbon Nanofibers</td>
<td>33</td>
</tr>
<tr>
<td>Structure and Manufacturing Procedures</td>
<td>33</td>
</tr>
<tr>
<td>Properties and Applications</td>
<td>36</td>
</tr>
<tr>
<td>CNTs/CNFs for Biomedical Applications</td>
<td>37</td>
</tr>
<tr>
<td>CNTs/CNFs for Bone Regeneration and Drug/Gene Delivery</td>
<td>37</td>
</tr>
<tr>
<td>CNTs/CNFs for Neural Applications</td>
<td>41</td>
</tr>
<tr>
<td>Toxicity of CNTs and CNFs</td>
<td>45</td>
</tr>
<tr>
<td>3 MATERIALS AND METHODS</td>
<td>48</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>48</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>49</td>
</tr>
<tr>
<td>Substrates</td>
<td>50</td>
</tr>
<tr>
<td>CNT Substrates</td>
<td>50</td>
</tr>
<tr>
<td>CNF, CTF, SU8NF and SU8TF Substrates</td>
<td>51</td>
</tr>
<tr>
<td>Imaging</td>
<td>53</td>
</tr>
<tr>
<td>Fluorescence Microscopy</td>
<td>53</td>
</tr>
<tr>
<td>AFM</td>
<td>53</td>
</tr>
<tr>
<td>SEM</td>
<td>54</td>
</tr>
<tr>
<td>Image Analysis: NeuriteQuant</td>
<td>54</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>58</td>
</tr>
<tr>
<td>4 RESULTS AND DISCUSSION</td>
<td>66</td>
</tr>
</tbody>
</table>
CNT Samples ........................................................................................................ 66
AFM and SEM Analysis ....................................................................................... 66
NeuriteQuant Analysis ....................................................................................... 66
Discussion ........................................................................................................... 68
CNF, CTF, SU8NF, SU8TF Samples ................................................................. 70
AFM and SEM analysis ....................................................................................... 70
NeuriteQuant Analysis ....................................................................................... 70
Discussion ........................................................................................................... 74
Comparison Between CNT and CNF Samples ............................................... 76

5 CONCLUSION AND RECOMMENDATIONS FOR FURTHER WORK .......... 102

LIST OF REFERENCES ......................................................................................... 105

BIOGRAPHICAL SKETCH ..................................................................................... 113
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Branched PEI molecule.</td>
</tr>
<tr>
<td>3-2</td>
<td>Drawing of a LBL sample.</td>
</tr>
<tr>
<td>3-3</td>
<td>Chemical structure of the Bisphenol A Novolak epoxy oligomer contained in SU-8 formulations.</td>
</tr>
<tr>
<td>3-4</td>
<td>Drawing of a CNF/CTF/SU8NF/SU8TF sample.</td>
</tr>
<tr>
<td>3-5</td>
<td>Conversion of the image from RGB type to 8-bit type using ImageJ.</td>
</tr>
<tr>
<td>3-6</td>
<td>Example of threshold setting with ImageJ.</td>
</tr>
<tr>
<td>3-7</td>
<td>Schematic representation of the algorithm used by NeuriteQuant for image analysis.</td>
</tr>
<tr>
<td>3-8</td>
<td>Image obtained at the end of NeuriteQuant analysis.</td>
</tr>
<tr>
<td>3-9</td>
<td>Primary and secondary measurements that can be obtained from NeuriteQuant.</td>
</tr>
<tr>
<td>3-10</td>
<td>Example of image modifications applied to CNF, CTF, SU8NF, SU8TF pictures before NeuriteQuant analysis.</td>
</tr>
<tr>
<td>4-1</td>
<td>AFM pictures of CNT(3LBL) and CNT(9LBL) samples.</td>
</tr>
<tr>
<td>4-2</td>
<td>SEM images of CNT(3LBL) and CNT(9LBL) samples.</td>
</tr>
<tr>
<td>4-3</td>
<td>Original pictures of neurons on CNT(3LBL) and CNT(9LBL) after 3 DIV and 7 DIV.</td>
</tr>
<tr>
<td>4-4</td>
<td>Original pictures of neurons grown on PEI(3LBL) and PEI(9LBL) at 3 DIV and 7 DIV.</td>
</tr>
<tr>
<td>4-5</td>
<td>Images of neurons cultured on CNT(3LBL) and CNT(3LBL) at 3 DIV and 7 DIV analyzed through NeuriteQuant.</td>
</tr>
<tr>
<td>4-6</td>
<td>Images of neurons cultured on PEI(3LBL) and PEI(3LBL) at 3 DIV and 7 DIV analyzed through NeuriteQuant.</td>
</tr>
<tr>
<td>4-7</td>
<td>Charts showing the values obtained from NeuriteQuant analysis for the percentage of live cells and the neurons’ cell body average size.</td>
</tr>
</tbody>
</table>
4-8 Charts showing the values obtained from NeuriteQuant for the neurite length referred to each neuron, the number of neurites per neuron and the average length of each neurite detected. ................................................................. 85

4-9 Charts showing the values obtained from NeuriteQuant analysis for the number of branches per each neurite and the number of branches for each neuron. .................................................................................. 86

4-10 AFM pictures of CNF, CTF, SU8NF and SU8TF samples................................. 87

4-11 SEM pictures of SU8NF and CNF samples....................................................... 87

4-12 Pictures of the stripes and the background of the four types of samples used... 88

4-13 Fluorescence Microscopy pictures of neurons on CNF, CTF, SU8NF, SU8TF samples only-plasma treated................................................................. 89

4-14 Fluorescence Microscopy images of neurons on CNF, CTF, SU8NF, SU8TF samples treated with 0.1% PEI................................................................. 90

4-15 Fluorescence Microscopy pictures of neurons on CNF, CTF, SU8NF, SU8TF samples treated with 0.001% PEI............................................................... 91

4-16 Fluorescence Microscopy images of neurons on CNF, CTF, SU8NF, SU8TF samples treated with 0.00001% PEI......................................................... 92

4-17 Bar chart representing the percentage of live cells over the total number of cells on the four types of samples that underwent four different types of treatments (plasma, 0.1% PEI, 0.001% PEI, 0.00001%PEI). ......................... 93

4-18 Bar chart representing the percentage of live cells over the total number of cells on the four types of samples that underwent four different types of treatments (plasma, 0.1% PEI, 0.001% PEI, 0.00001%PEI). ................. 94

4-19 Bar chart showing the results related to neuron’s cell body average size on CNF and CTF obtained from NeuriteQuant................................................. 95

4-20 Bar chart representing the data obtained from NeuriteQuant analysis in relation to the neurite length per cell, the number of neurites per cell and the average length of each neurite .............................................................. 96

4-21 Bar chart showing the number of branches per cell and the number of branches per neurite of neurons plated on CTF and CNF treated with plasma, 0.1% PEI, 0.001% PEI and 0.00001% PEI, respectively............. 97

4-22 Bar charts representing the percentage of live cells and the cell average size obtained from the NeuriteQuant analysis performed on neurons cultured for 3 DIV on CNT and CNF samples......................................................... 98
4-23  Bar chart representing the values obtained for the neurite length per cell, the number of neurites per cell and the average length of individual neurite of neurons grown for 3 DIV on CNT and CNF samples........................................ 99

4-24  Bar chart showing the number of branches per neurite and per cell of neurons cultured on CNF and CNT samples for 3 DIV obtained from NeuriteQuant analysis ........................................................................................................ 100

4-25  Pictures after NeuriteQuant analysis of neurons at 3 DIV on CNT(3LBL), CNT(9LBL), CNF plasma treated and CNF treated with 0.00001% PEI......... 101
In this work we investigated viability and neurite outgrowth of neuronal cells onto topographically and chemically modified surfaces. In particular purified carbon nanotubes (CNTs), electrospun SU8 carbonized (CNFs) and non-carbonized nanofibers (SU8NFs), flat stripes of SU8 thin film carbonized (CTFs) and non-carbonized (SU8TFs), and polyethylenimine (PEI) have been used, individually or in combination, as surface modifications. CNTs have been applied onto glass coverslips through a layer by layer (LBL) deposition method in which layers of PEI and CNT solution were deposited alternated on the surface. Varying the number of layers, we fabricated two types of CNT-samples: 3 layers (3LBL) and 9 layers (9LBL). All CNT samples were plasma treated after manufacturing. On the other end, the CNFs, SU8NFs, CTFs and SU8TFs were deposited on silicon substrates, plasma treated and coated with PEI at different concentrations, 0.1-0.001-0.00001 %. Dissociated E18 rat cortex neurons have been seeded on these substrates. Using calcein AM and ethidium bromide (EthD-1), cells have been stained and fluorescence microscopy applied. The images obtained were processed using NeuriteQuant, an ImageJ plugins, for cell counting and neurite extension measurements. Cell viability and neurite outgrowth was found to be the same
on CNT(3LBL) and CNT(9LBL) samples but slightly inferior to PEI control samples. For the CNFs, SU8NFs, CTFs and SU8TFs tested, very few cells grew on them at concentration of 0.1 % PEI instead high viability and neurite outgrowth was found on these samples only plasma treated or coated with 0.00001 % PEI. Moreover, from the neurite analysis, we observed that CTF and CNF samples supported very good neurite development and branching showing that their application as coated materials for electrodes could be a successful solution in order to decrease the impedance and favor neuronal growth.
CHAPTER 1
INTRODUCTION

Current techniques for neural recording and stimulation require improvements in electrode manufacturing in order to decrease their impedance and improve their conductive properties so that robust signal recording or delivering can be achieved.

Microelectrode arrays (MEAs) are used for both in vitro and in vivo purposes. In vivo they have been used as electrodes in deep brain stimulation (DBS) to treat chronic pain, Parkinson's disease, tremor and dystonia, in cochlear implants and in cardiac pacemakers.

In vitro recording and stimulation, through MEAs, are considered very important in order to understand how the neural network works and then use this acquired knowledge as guidelines and theoretical basis for neural tissue regeneration. In vitro, recording of spontaneous neural activity as well as stimulation can be performed on both dissociated neurons and neural tissue slices.

In both in vivo and in vitro applications, the quality of recorded and delivered signals depends on the electrode properties (e.g. geometry, impedance, noise) and on the contacts established between the cells and the electrodes. Physics suggests that, in order to decrease the impedance, the electrode surface area should be increased. On the other side, the electrodes should be as small as possible to allow recording and stimulation with high specificity. To satisfy these two contradicting factors, chemical and topographical modifications are needed to create electrodes with high specific areas. Moreover neurons are cells far more particular than most cell types and special surface treatments are needed, prior to culturing, to facilitate cell adhesion, proliferation and growth.
As described by Ben-Jacob and Hanein [1] carbon nanotubes (CNTs) can be the perfect solution that can deal both with the need to decrease the impedance of the electrode and to improve cell adhesion. CNTs have very good mechanical and electrical properties and several studies, which will be later discussed, have also confirmed their biocompatibility. Moreover they create a rough surface in the nanometer scale that can potentially mimic the cells natural environment created by the extra cellular matrix.

Carbon nanofibers (CNFs) have properties very similar to CNTs but bigger diameters so that they can increase the surface roughness even more. In addition, different types of chemical modifications can be used to improve cell attachment. Polyethylenimine (PEI), polylysine (PLL) and polyethyleneglycol (PEG) are just three of the most investigated polymers used in neural cell culture.

In this study, for future applications of CNTs and CNFs coating directly on the MEAs, we investigated how the cell viability and neurite outgrowth can be affected by such materials coupled with PEI and applied to glass coverslips and silicon. Moreover we also investigated how cells behave on uncarbonized electrospun nanofibers (SU8NF) made of SU8 polymer in order to understand if a bigger nanofiber diameter (two fold the CNF diameter) and the absence of a carbon-structure can still allow neuronal adhesion and development. In parallel with SU8NFs we cultured neurons on flat SU8 stripes, either carbonized or uncarbonized, to understand at which extent the chemical composition of the photoresist influences cell adhesion and how does that factor change when the stripes undergo a carbonization process.

The work is organized into four main chapters: background, materials and methods, results and discussion and conclusions. The background is divided into three
sections discussing, respectively, studies related to chemical and topographical surface modifications and neurons interactions, CNTs and CNFs, and applications of CNTs and CNFs in tissue regeneration. In the materials and methods are presented all the techniques and procedure used for the study. In its sections will be described cell culture methodology, substrates manufacturing techniques as well as imaging instrumentation and software used for image analysis. Finally experimental results together with their possible interpretations are presented in the second to last chapter. In the concluding chapter a brief summary of the results obtained is presented together with analysis and investigations that might be useful to perform in the future.
CHAPTER 2
BACKGROUND

Neuronal Cell Growth and Surface Modifications

Neurons differ from every other type of cell due to their specific morphology and their signal transmission functions. They are polarized cells with generally short receptive extensions (called dendrites) on one end and a singular long extension (called axon) with synaptic terminals on the other with high selectivity to the substrate [2]. Several studies on neural growth have clarified how neurons develop in order to acquire this specific structure and connect with each other. The environment around and below them has been defined as one of the key aspect that regulate the spatial organization of the neurons’ cytoskeleton. The latter undergoes several changes in relation to external cues (often called “contact guidance”) encountered. In the central nervous system, these cues are offered by glia cells that guide the filopodia and lamellipodia of the growth cone (an expanded terminal structure at the end of the axon shaft that allows cellular development). Moreover, the adhesion, spreading, migration, proliferation, and differentiation of neurons are driven, in the natural tissue, by the topographic cues of the extracellular matrix (ECM) [2].

In vitro neuronal cell growth investigations are very important in order to understand neurons’ behavior on specific type of surfaces that differ from the natural one. Sometime, in order to study cell-biomaterial interactions, surface modification is considered a highly significant investigation tool. In vitro analysis can be conducted seeding neuronal cells on chemically and topographically modified surfaces to evaluate cell adhesion, proliferation, apoptosis, migration, orientation, and differentiation. Moreover, specific type of modifications can better mimic the neural cells’ environment
(i.e. extra cellular matrix (ECM)) so that in vitro cell behavior can be easier related, if not compared, to in vivo cell performance. To better understand the possible surface modifications that can be applied, an overview of the materials commonly used as substrate will be presented. Moreover, in order to make the neurons adhere on these modified surfaces coating materials have to be used. An overview of these coating materials and of the possible coating techniques to be used will be also presented. In conclusion, several studies on surface modifications for neural cell growth will be described.

**Substrates for Neural Cell Culture**

Normally substrates for biomedical applications and in vitro analysis are made of: flat glass, polystyrene, sapphire, iridium oxide, silicon, metal oxides, polymers, and platinum [2]. They have been used either unmodified or with surface modifications. Among the materials used for in vitro cell culture, silicon is one of the most studied mainly for its chemical and physical properties that make it suitable for implantable device fabrication. Several experiments have shown cells growing healthy on silicon samples. Moreover, it has been proved that, surface modifications to silicon, significantly increase neuron adhesion in vitro.

Metals like platinum (Pt), tungsten (W), stainless steel, indium, titanium nitride (TiN), Pt/W and Pt/Ir have been widely used as substrate for DBS (deep brain stimulation) electrode-fabrication and microelectrode arrays [2]. Micro and nano patterning of these metals can have important advantages on both electrical conduction and cell adhesion. For instance, Cyster et al. [3] investigated hippocampal neuron attachment on titanium nitride film samples. They fabricated samples with different
roughness and neurons showed a better preference for surfaces with lower roughness and decreased size of topographical features.

Polymers like PMMA, PDMS, polystyrene, biodegradable polymer and conductive polymer are commonly used as substrates for neural cell patterning. Among all the techniques used for polymer surface modifications there are: soft lithography, polymer casting, photopolymerization, micro-contact printing, microfluidic, and stencil patterning.

Carbon nanotubes and carbon nanofibers can also be used for surface topographical modifications and a large part of our work will be specifically dedicated to this topic.

**Coating Materials for Neural Cell Culture**

Coating materials are very important in neural cell culture as neurons do not attach by themselves to several of the above described substrate-materials. In order to study the effect of topographical cues on neuronal cell growth, adhesion-promoting coating materials (either natural or biologically active/inactive or synthetic) have to be used. ECM proteins and cell adhesion molecules (CAM) are among the most used natural materials that promote adhesion by receptor-mediated binding. Biologically inactive or synthetic materials are commonly positively charged polyaminoacids, such as polylysine, or amino functional groups attached to silanes and thiol-linking groups [2].

Among the ECM proteins, laminin, collagen, and fibronectin are the three most investigated. They have multiple binding sites for cell surface receptors that can anchor the neurons to the substrate onto which they are applied [4].

Polylysine, polyornithine (polyamino acids) and polyethylenimine (polycation) are very common polymers to be used as neural cell-attachment-promoters on glass or
plastic. The driving principle for them to improve cell adhesion is the interaction between their positive charge and the negative charge of the phospholipids composing the neuronal cell membrane [5]. This type of molecular adsorption of the polymer onto the surface is commonly referred as physisorption.

On the other end, chemisorption takes place when strong chemical bonds form between the molecules applied on the surface and the surface itself. In such a way self-assembled monolayers (SAM) can be obtained using organic molecules (both aliphatic and aromatic) containing free anchor groups (heads) such as thiols, disulphides, amines, silanes, or acids that bind to the surface [2]. The head groups are connected to an alkyl chain in which the terminal end can be functionalized (for instance adding –OH or –NH\textsubscript{3} or –COOH groups) to improve cell adhesion. One of the most common studied methods to bind SAM to the surface is using thiolate-based monolayers on gold. Moreover, organosilane can also be used to produce molecular patterns. Stenger et al. [6] showed that, through organosilanes patterns, adhesion of rat hippocampal neurons could be controlled and neurite outgrowth could be spatially directed.

When the substrate’s surface is hydrophobic there is the need to improve the hydrophilicity of the material so that cells can better adhere to the surface. In order to do that, specific functional groups have to be placed on the surface either through chemical modification or coating surface procedures. Anionic hydrophilicity can be obtained with carboxy group (–COO\textsuperscript{−}) whereas neutral hydrophilicity can be obtained with hydroxy (–OH) or amide (–CONH) groups and cationic hydrophilicity with amino groups (–NH\textsubscript{2}, –NHR, –NR\textsubscript{3}) [2]. Plasma treatments can also be very useful in order to improve surface hydrophilicity.
Studies on Surface Modifications and Related Neurite Growth

Two types of surface modifications are possible: topographical modifications, in the micrometer or nanometer scale, using different manufacturing techniques (i.e. photolithography, wet etching, reactive ion etching and laser fabrication) or chemical modifications using various protein or cell adhesive molecules that change the chemical composition of the surface [2].

Surface topographical modifications can be divided into two main types: anisotropic (such as ridges and grooves) and isotropic (for example evenly or randomly distributed pits or protrusions). The first type has been applied mainly to investigate if the neural growth could be guided along the anisotropic direction independently from the nano- or micro-scale of the modified structure. The second type has been used preferentially to investigate cell adhesion variations in relation to the structure-scale [7].

According to neuronal cell size and axon diameter, 5 to 20 µm and 1 to 20 µm respectively, micrometer scale patterning can be useful to study cell surface interactions whereas nanometer scale can be more suitable in order to investigate how surface modifications can influence neurite growth. Conventional photolithography has been used for micrometer surface modifications in the range of 30-100 µm whereas, using deep UV, it is possible to get feature size smaller than 30 nm. Electron beam lithography has been applied to create features in the range of 1–2 µm [2]. Chemical etching is another technique for surface modification that allows the creation of a random roughness surface of 2–800 nm range. Surface modifications can be performed on polymeric substrates using nanoimprint lithography that allows the formation of features down to 5 nm. Laser-assisted nanopatterning combined with SPM has been used to create nanotopographical surfaces. Chemical vapor deposition allows carbon
nanotubes formation whereas, from electrospinning, nanofibers (and then carbon nanofibers) can be obtained. In the next chapters these last two techniques will be discussed extensively.

Several investigations have, as their main goal, to understand how cells react and behave on specific type of patterns like grooves, ridges, steps, pores, wells, nodes, pillars, islands, and adsorbed protein fiber [2]. Here we will discuss some of these studies involved with neuronal cells, underlining the substrate/coating materials and techniques applied as well as the specific type of neuronal cells used and the results obtained.

Different types of silicon substrates have been studied by Bayliss et al. [8] in order to understand how nanostructured silicon can improve neurons adhesion. They compared porous silicon (PS) substrates (with pore size of 10 nm) to both PECVD polycrystalline silicon and bulk silicon substrates on the basis of rat neural B50 cells growth. Cell adhesion and viability values were much higher on PS substrates compared to the other substrates. Fan et al. [9] fabricated through etching procedure various nanostructured silicon substrates to test neurons adhesion and viability. According to their results, cells survived for over 5 days with normal morphology on samples with an average roughness of 20-50 nm. As a contrary, cell adhesion was adversely affected on surfaces with extremely low roughness values (less than 10 nm) or on surfaces too rough (roughness higher than 70 nm).

Lee et al. [10] studied SiO₂ patterned surfaces for cell growth. Three microtrack patterns (linear, dashed, and zigzag) with a height of 4 μm where created through conventional photolithography and etching processes. From their analysis, zigzag type
pattern was shown as the most effective for directional guiding of neuronal growth and motility.

Grooved substrates have been investigated as surfaces for neural cell growth by Clark et al. [11] [12] and Rajnicek et al. [13]. The first group tested how chick embryo cerebral neurons grew and developed on quartz substrate with grooves of 130 nm width and various depths (100, 210 and 400 nm) separated by 130 nm space realized with laser holography and microelectronic fabrication techniques. They found out that the neurite outgrowth was not affected by such a pattern [14]. As a second attempt, the same group studied neurite outgrowth of chick embryo cerebral neurons on Poly(methyl methacrylate) (PMMA) coated with poly-L-lysine. They patterned the substrates using photolithographic and dry etching techniques to obtain grooves 8 μm wide and 20 μm spaced with different depths (1 or 2 μm). Their results showed that depth can play an important role in neurite alignment as 1 μm depth does not affect neurite orientation whereas on grooves 2 μm deep the cells grew following groove’ directionality [15].

The second group analyzed rat hippocampal neurons development on micropatterned substrates realized with fused quartz using a direct writing electron beam lithographic process. The substrates were patterned with grooves of different width and depth and the results obtained showed that hippocampal neurites grew parallel to deep and wide grooves (1100 nm depth, 4 μm width) but perpendicular to shallow and narrow ones (130 nm depth, 1 μm width) [16].

Turner et al. [17] [18] investigated how neurons and astrocytes (both from LRM55 astroglia cell line) developed on silicon pillar- and well- patterned surfaces. Both cell types preferred to grow on pillars independently from their height and distances (the
authors estimated that 70% of the cells were found wrapped around the pillars). The same thing did not happen with well-patterned surfaces were just 40% of the cells preferred wells to flat surface.

Gomez et al. [19] showed important results on axogenesis and axon growth mechanisms. They fabricated poly(dimethylsiloxane) (PDMS) substrates with topographical (microchannels) and chemical (Nerve growth factors (NGFs) bound to specific surface’s areas) modifications presented individually or in combination. Polarization (i.e., axon initiation or axogenesis) was shown to be positively affected by topographic cues whereas NGFs did not seem to have any effect on it. On the other end, on NG-modified areas, axons grew more compared to channels areas and the combination of the two cues (topographic and chemical) seemed to synergistically increase axon length even more. Moreover, axon orientation was also analyzed varying the width (1 μm and 2 μm) and depth (400 nm and 800 nm) of microchannels. They confirmed the tendency of cells, as noted in other studies cited above, to grow perpendicular to narrower and shallower microchannels (1 μm width and 400 nm depth) and parallel to wider and deeper microchannels (2 μm width and 800 nm depth) [20]. In another work from the same group [21], polypyrrole (PPy), an electrically conducting polymer, has been micropatterned into 1 and 2 μm wide microchannels using electron-beam (e-beam) lithography and electropolymerization. Embryonic hippocampal neurons cultured on patterned PPy were able to polarize (i.e., form an axon) faster compare to unmodified PPy with a significant increase in the number of cells with axons. Orientation of the axons was shown to be positively affected on modified PPy whereas the overall axon length was comparable to the one measured on unmodified PPy [22].
Kaehr et al. [23] created microstructured surface modifications directly in situ during cell growth. They used multiphoton excitation to focally excite noncytotoxic photosensitizers that promote protein crosslinking, such as bovine serum albumin (BSA), into 3D matrices having feature sizes bigger than 250 nm. Neuroblastoma-glioma (NG108-15) cells were cultured on poly-L-lysine-coated glass coverslips and exposed to a solution containing BSA in order to fabricate in situ 3D microstructures. As a result, neurites actually modified their orientation over time following the patterns of these protein matrices.

Studies on nano-printed patterns on polymethylmethacrylate (PMMA)-covered silicon chips have been carried out by Johansson et al. [24]. The patterns consisted of parallel grooves with depths of 300 nm, varying widths of 100–400 nm and distance between two adjacent grooves of 100–1600 nm. Adult mouse sympathetic and sensory ganglia were mounted on these samples and cultured with medium containing NGFs. They found that axons and dendrites grew following the pattern in all the samples and, moreover, that neurites preferred to grow on ridge edges and elevations rather than in grooves [25].

Several studies dedicated to CNTs and CNFs surface modifications are discussed in detail in the following chapters.

Topographical and chemical modifications have been tested also on microelectrode arrays (MEAs) in order to confine neurons into highly defined patterns and be able to control their local density. Chang et al. [26] modified an electrode array surface creating, through lithographic technologies, a pattern consisting of alternating 40 μm wide lines of poly-D-lysine (PDL) and 60 μm wide lines of silicon nitride.
After 27 DIV embryonic hippocampal cells aligned on the PDL stripes and they found that the apparent electrical activity of the network, measured from the underlying electrodes, increased 8–10-fold with greater local density [26]. Nam et al. [27] formed, through microstamping, micrometer scale patterns of cell adhesive proteins using (3-glycidoxypropyl) trimethoxysilane (3-GPS) on MEAs. Hippocampal neurons after 1 month DIV followed those patterns. Such a surface modification allowed spontaneous neural activity to be recorded as early as 10 days in vitro, a much shorter time compared to unmodified MEA where two to three weeks are required before been able to detect proper cell activity [27].

**Carbon Nanotubes**

CNTs have been used as surface modifications with very significant results. Here are presented some background information about CNTs and CNFs manufacture and properties in order to understand why so much attention has been directed to them in the past few years and why they can be so important in neural engineering applications.

**Structure and Manufacturing Procedures**

The discovery of Carbon Nanotubes is due to Sumio Iijima, an electron microscopist from the NEC laboratories in Japan, who was inspired by the work of Krätschmer and Huffman. The latter scientists published a paper in *Nature* in 1990 about the Buckminsterfullerene, a closed cluster made of 60 carbon atoms (C$_{60}$) organized on pentagonal structure, that was shown to be the most common and stable configuration found in the soot of carbon arc vaporized graphite [28]. Sumio Iijima repeated the same experiments carried out by Krätschmer and Huffman and, focusing his attention on the graphite cathode rather than on the wall soot produced by arc-evaporation, he found out long and fine hollow fibers that took the
name of Carbon Nanotubes (CNTs). CNTs are tubes closed at both ends with caps which contain pentagonal carbon rings and can be interpreted as half of the buckminsterfullerene. The main body of the CNT is formed by hexagons of carbon atoms and, according to their arrangement around the circumference, CNTs can be classified as ‘zig zag’, ‘armchair’ and ‘chiral’ CNTs [28].

Moreover, CNTs can be classified as Multiwalled Carbon Nanotubes (MWCNTs) with the inner diameter in the range of 1.5-15 nm and the outer diameter in the range of 2.5-30 nm, Single-walled Carbon Nanotubes (SWCNTs) with a diameter in the range of 0.7-1.6 nm and Double-walled Carbon Nanotubes (DWCNTs). MWCNTs and DWNTs are made by two or more layers that can be organized into a “Russian doll” or a “Swiss roll” as shown of Figure 2-8. Single-walled CNTs are made by just one graphitic layer, a structure that allow us to define them as the most ‘ideal’ nanotubes compared to the other categories.

CNTs can be synthetized through two main methodologies that have been highly improved over the years.

Arc-vaporization has been used since the first experiments conducted on CNTs. The classic Arc-vaporization is carried out using a chamber connected both to a vacuum line with a diffusion pump, and to a He supply. The pressure of the He has to be kept at 500 torr, the optimum pressure for CNT production. Two graphite rods are used as electrodes where the anode is a long rod of approximately 6 mm in diameter and the cathode is a much shorter rod of 9 mm in diameter. Moreover a voltage-stabilized DC power supply is normally used, and discharge is typically carried out at a voltage of 20V. The current is usually in the range 50–100A and has to be kept as low
as possible in order to obtain a high number of free nanotubes. At the beginning of the
process, the two electrodes should be taken far apart and slowly moved closer until
arcing occurs. Normally the rod is consumed at a rate of a few mm per minute and
whenever it is totally consumed the process end. At this point on the cathode a
cylindrical deposit will be found, consisting of a hard outer shell of fused material and a
softer fibrous core with nanotubes and nanoparticles. Other type of gas as other
materials for the rods have been recently tested and used in this methodology. In
particular it has been proved that, in order to promote the synthesis of SWCNTs, Fe,
Co and Ni must be added on the surface of the electrodes keeping the Helium pressure
at around 500–800 torr. DWNTs can be obtained using as catalyst a prepared mixture
of Ni, Co, Fe and S and, as gas, a mixture of Ar and H$_2$ (1:1) at 350 torr [29].

Catalytic Chemical Vapor Deposition (CCVD) is another methodology for CNT
synthesis. This process allows a larger-scale production compared to arc-vaporization
and, at the same time, due to the mild conditions and the possibility to control over the
growth process, it allows to grow CNTs following a specific pattern or array. Nano-
electronic circuits have also been produced using CCVD. As a drawback, MWCNTs-
CCVD-produced are considered structurally inferior to arc-vaporization-MWCNTs even
if it has been shown that CCVD-produced SWCNTs can have the same or even higher
degree of structural perfection compared to arc-vaporization-SWCNTs. In this process,
a patterned catalyst (Fe, Co or Ni) is deposited in the form of nanoparticles onto the
surface of the substrate that is held in a furnace at an initial temperature of
approximately 1000 °C (1200 °C for SWCNTs). Specific types of gases (i.e. High-purity
hydrogen, benzene) flow across it and CNTs are synthesized. By controlling the
nanoparticles’ size, the CNTs dimensions can be determined. Another very popular approach for CNT growth involves the use of a plasma. A plasma is an excited/ionized gas, and the processing plasmas, usually known as ‘cold’ plasmas, are generated using DC, RF or microwave excitation. Plasma Enhanced Chemical Vapor Deposition (PECVD) does not involve high temperatures and is widely used to produce aligned nanotubes [30].

CNTs produced from both methodologies above described will never be pure carbon nanotubes. For this reason, there is the need for specific purification processes that remove the arc-vaporization or CVD by-products. At the moment there are no completely satisfying procedures to purify arc-synthetized CNTs. The most commonly applied are involved with oxidation of nanoparticles and other graphitic contaminants that can be found among the produced CNTs. The main problem related to this technique is that reactivities of CNTs and nanoparticles towards oxidation are very similar so that the process must be highly controlled in order to avoid complete oxidation of the CNTs that are to be preserved. Solubilization methods have also been applied to purify arc-synthesized MWCNTs. In order to purify CVD-produced CNTs catalyst’s residuals have to be removed. This procedure is much easier compared to nanoparticle removal for arc-synthesized CNTs. Thermal annealing is considered one of the most effective procedures as not only the catalyst impurities can be removed but also the structure of the CNTs can be highly improved. Acid treatments can also be applied but damage to CNTs’ structure have been often shown [31].
Properties and Applications

The importance that CNTs has increased since their discovery is mainly due to their excellent electronic and mechanical properties. Moreover, optical and thermal properties add even more interest in finding useful applications of these nanostructures.

CNTs can be classified as metallic or semiconducting in relation to their structure. In particular, from the work of Mildred Dresselhaus and co-workers from MIT and Noriaki Hamada and colleagues from Iijima’s laboratory in Tsukuba, it can be stated that, among SWCNTs, all armchair configurations are expected to be metallic while approximately one-third of zigzag and chiral tubes should be metallic with the remainder being semiconducting [32][33][34].

Metalllic SWCNTs’ behavior has been classified as a quantum wire behavior and this explains by itself the extremely high relevance of their electronic properties. A quantum wire is a structure where the conduction happens through well separated and discrete electron states. Therefore the resistance does not change along the wire as it would happen in a macroscopic structure. Moreover the quantum behavior in metallic CNTs is ballistic in nature so that no scattering, due to impurities or phonons, that place. In other words, CNTs can conduct a large current without getting hot. However, metallic SWCNTs cannot be considered as super-conductors because the conductance quantum in this case is not infinite [35].

In semiconducting SWCNTs, the behavior seems to be diffusive rather than ballistic even if the precise nature of the scattering processes has not been fully established yet [35].

Due to these properties, several investigations have been carried out on possible application in nanoelectronics. For instance the fabrication of diodes, Field Effect
Transistor (FET) and logic circuits using CNTs has been receiving fast growing attention[35].

The mechanical properties have been analyzed by several authors using TEM, AFM and SEM measurements and, as a result, MWCNTs arc-synthesized have been shown to have an average Young’s modulus of 1000 GPa, almost five times higher than steel, and a tensile strength up to 63 GPa, around 50 times higher than steel. For this reason P. Harris stated in his work that “CNTs are the stiffest and strongest fibers ever produced” [36]. On the other end studies on MWCNTs produced through CVD have shown lower mechanical properties with a Young’s modulus in the range of 100-350 GPa mainly due to the higher presence of defects in their structure[37].

Due to their good mechanical properties, CNTs are currently applied in the manufacturing of AFM tips with very good results in terms of imaging qualities. The prices are still high so that they are not commonly used but they will acquire much more popularity when CNTs manufacturing procedures will be standardized and made less cost effective [38].

Only few measurements have been carried out on individual SWNT do to the difficulties and challenges encountered. Ropes of CNTs are easier to be analyzed and several authors have been using them to calculate the Young’s modulus and tensile strength of individual SWNT that, on average, are equal to 1.0 TPa and 30GPa respectively [39]. Surprisingly the same results have been confirmed for both CVD- and arc-synthesized SWCNTs proving that their mechanical properties can be considered independent from the preparation method [36].
CNTS optical properties are mainly related to the strong optical absorbance of SWCNTs in the NIR spectral window (~800–1600 nm) as shown by Kam et al. [40]. These emission peaks in the near infrared, first investigated by O’Connell et al. [41], were actually attributed to fluorescence across the band gap of semiconducting nanotubes. Therefore fluorescence can be used to define CNTs structure but it is noted worth to say that only semiconducting nanotubes can actually exhibit fluorescence so that roughly one-third of CNTs have to be excluded [36].

The thermal conductivity has been characterized mainly for MWCNTs and, for arc-grown tubes, values greater than 3000Wm\(^{-1}\)K\(^{-1}\) at room temperature were found. Instead, the thermal conductivity of catalytically-produced MWCNTs appears to be much lower [42].

**Functionalization Procedures and Applications**

CNTs are well known for their hydrophobicity that makes them highly insoluble and water repellent.

In order to make them hydrophilic, functionalization can be carried out. Several methodologies and techniques can be applied to achieve these results. Functionalization can be performed modifying the CNTs surface using chemical groups, polymers, or surfactants.

CNTs can be modified with positively or negatively charged chemicals by covalently incorporating various reactive groups such as \(-\text{NH}_2\), \(-\text{SH}\), and \(-\text{COOH}\) [43]. Further functionalization can be achieved by reaction with these groups.

A “non-covalent” approach involves the use of polymers or surfactants to change CNTs hydrophobic properties and, in biological applications, to make them more biocompatible and amenable to cell surface interactions. It has been proved that a
positively charged surface may be particularly important for regenerative medicine applications, because it enhances electrostatic interactions with the negatively charged plasma membrane of neural cells. As commonly used polymers, such as polyethyleneimine (PEI) and poly-L-ornithine (PLO), promote neural cell attachment and subsequent neurite outgrowth, CNTs modified to be positively charged are a promising substrate for neural cells when applied as a coating material on neural interface devices. Functionalization can also be performed using hydrophilic polymers such as polyethylene glycol (PEG) which increases CNT solubility in aqueous solution as well as biocompatibility [43].

Biologically relevant and effective molecules can also be used in the functionalization process. The biologic factors of interest that might be linked to CNTs include therapeutic drugs, proteins and genes. This strategy, when used in cell culture applications, does not just overcome cellular toxicity but can also improve or increase specific cell functions such as cell attachment, neurite outgrowth, neuronal differentiation, and nerve tissue regeneration [43].

The extremely large surface areas and the various functionalizations that can be applied, make the CNTs suitable for gas sensor manufacturing. Moreover, the conventional solid-state sensors operate at temperatures over 400 °C whereas nanotube-based sensors can operate at room temperature. Even if several investigations have been carried out, more time is needed for their commercialization [38].

Another application of CNTs is in the biochemical field as macro- or nano-electrochemical biosensors. In the macroscale, biochemical sensors are usually made
of three electrodes, a reference electrode, an active electrode and a sink electrode. The active electrode detects the analyte generating a reaction that releases specific ions. The latter creates a potential that is subtracted from that of the reference electrode. This difference will give rise to a signal. The high surface area and conductivity of CNTs make them extremely suitable for electrode applications. In the nanoscale, SWCNTs have been investigated as a potential detection system during protein formation. Basically CNTs have been directly grown or applied on a field effect transistor (FET) configuration and functionalized so that specific receptors are attached to the coated tubes. Whenever a specific reaction was taking place the current was measured and the changing in the conductivity of the CNTs could be easily related to that specific reaction. As an example Besteman et al. [44] immobilized an enzyme, a glucose oxidase (GOx), on the SWCNTs via a pyrenyl group linking molecule. The conductivity of the GOx-coated tubes was found to change with pH and with addition of glucose, demonstrating that single nanotubes can act as sensors [38].

**Carbon Nanofibers**

**Structure and Manufacturing Procedures**

Carbon nanofibers can be divided into two main categories: Vapor-Grown Carbon Fibers (VGCF) and carbonized polymer fibers. VGCFs differ from CNTs mainly for the diameter (ranging from 50 to 100 nm for CNFs) and the orientation of the graphitic layers in relation to the fiber axis. Martin-Gullon et al. [45] identify six different types of CNFs.

Platelet-CNFS, with a width of 100 nm, are made of several layers of graphene, perpendicular to the axis of the nanofiber with the catalyst particle in the middle. They can have also a spiral orientation. CNFs are defined as Fishbone-CNFS when the
graphene layers have a specific inclination with respect to the fiber axis. They can have a hollow or solid core but, in both cases, hydrogen is required to stabilize the edges in the structure. Ribbon-CNFS are made of straight, unrolled graphene layers parallel to the fibril axis with non-cylindrical cross-sections. Stacked Cup-CNFS are comprised of a continuous layer of rolled graphene along the fiber axis, hollow inside. If these types of CNF are coated with a layer of amorphous carbon non-catalytically CVD produced they become “thickened nanofibers”.

VGCNFs are synthetized from small metal particles (made of iron, nickel, or cobalt), as catalysts, exposed to CO or hydrocarbon gases, as carbon feedstock, at temperatures between 500 and 1200°C. A fibrous carbon structure, made of carbon atoms from the decomposed gas, starts growing around the circumference of the particles so that its diameter will be directly connected to the particles’ diameter. The orientation of the graphitic planes in the fiber depends on the crystallographic orientation of the catalyst particle [46].

Among the metal particles commonly used in order to grow CNFs, it has been proved that Fe yields platelet-CNFS at low temperatures (ca. 700 K) and ribbon or ‘tubular’ CNFs at higher temperatures (950 K) whereas Ni catalyst yields mostly fishbone nanofibers (both solid and hollow) and, at very specific conditions (big particles, temperature above 900 K), to platelet-CNFS [45].

In order to reduce the costs and simplify the manufacturing processes, CNFs’ production by stabilizing, carbonizing and activating electrospun precursors has been extensively investigated in the literature. Electrospun CNFs can have a diameter in the range of 100-1000 nm. Electrospun precursors are made of polymeric materials
electrospun in order to acquire a nanofiber shape. Carbonization is then performed on the nanofibers in order to obtain CNFs.

Electrospinning is a fabrication process in which an electrical potential is applied between a drop of polymer solution held at the tip of a needle and a grounded collector [47]. As the droplet grows, more charge accumulates at the tip of the needle and the electrostatic repulsion forces, due to the electric field applied, counteract the surface tension and the formation of the so called “Taylor cone” occurs. This cone continues to elongate until the electrostatic forces become larger than the surface tension. At that point a liquid jet is initiated from the tip of the cone that first grows longer and thinner and then, due to the change from ohmic to convective flow during the solidification process, the jet starts bending until it reaches the collector producing a web of non-woven fibers [48].

Electrospun nanofibers are usually smooth and solid fibers in the 100-600 nm diameter range. Nonetheless, using a different electrospinning setup (for instance two coaxial capillaries as spinneret) as well as different types of polymer, core-shell structure, hollow structure and porous nanofibers can be obtained.

Carbonization is the process that converts the electrospun NFs into CNFs. It is performed by heat treatments in a furnace with temperature in the range of 800-2000°C. During carbonization a significant weight loss and shrinkage occur to the NFs, resulting in the decrease of fiber diameter [49].

More than 100 types of polymer have been used in the past 20 years to produce electrospun NFs but only a few have been converted into CNFs. The most investigated
polymers to produce CNFs are polyacrylonitrile (PAN), polyimide (PI), poly(vinyl alcohol) (PVA), poly(vinylidene fluoride) (PVdF), SU-8 and pitch [49].

**Properties and Applications**

VGCNFs share the same mechanical and electrical properties as CNTs even if the different type of structures that they can assume present more imperfections so that part of these properties can be lost.

CNFs obtained from electrospun fibers have mechanical, electrical and chemical properties that highly rely on the nanotexture formed during carbonization process. In relation to the preferred orientation of the basic structural units of carbon layers, the nanotexture is classified into random, planar, axial and point orientations. Moreover, in relation to their precursors, carbon materials have been classified into two groups: graphitizing (a three-dimensional ordered structure that can be easily obtained at temperatures higher than 2500°C) and non-graphitizing carbons (a three-dimensional structure, hard to obtain even at high temperature) [49]. When the CNFs develop a graphitic structure, conductivity and therefore electrical properties can be highly improved. Usually heat treatments are very effective in developing graphitic structured-NFs. Among the most common type of electrospun CNFs, PAN-derived carbon fibers prepared via melt-spinning, stabilization and carbonization have a random nanotexture and are non-graphitizing. In order to improve their structure and make it axial oriented, stretching of the fibers is needed before further heat treatments. When aromatic polyimides (PIs) are used as carbon precursors, a wide range of CNFs’ structures can be obtained in relation to which combination of dianhydride and diamine is chosen [49].

Electrospun carbon nanofibers are usually prepared in the form of webs (mats) where different kinds of pores are formed: large pores (that we can even call spaces),
macropores ( > 50 nm size), mesopores (2–50 nm size) and micropores ( < 2 nm size). The presence of these macropores is very important for CNFs applications as, for instance, in energy storage devices they are pathways for electrolytes to diffuse into smaller pores to be adsorbed. Therefore the control of pores’ size and quantity is extremely important because a too large amount of pores can make the volumetric storage capacity small [49].

There is a large number of possible applications of CNFs in electronic devices fabrication and just few examples are high-power electrochemical capacitors as well as applications in lithium-ion batteries and in fuel-cell electrodes. Moreover their mechanical properties are exploited in the enforcement of different type of materials for construction purposes as well as in the realization of nanocomposite materials. As for CNTs, gas storage through CNFs has attracted a great deal of attention. CNFs have also been used and tested as catalyst support materials [46].

**CNTs/CNFs for Biomedical Applications**

Tissue engineering is a rapidly growing field. The need for new materials with high qualities to be applied in tissue regeneration has determined tentative applications of CNFs and CNTs to enrich the materials commonly used. Here, CNTs and CNFs applications on bone regeneration and drug delivery will be presented. Moreover the attention will be focused on neural applications.

**CNTs/CNFs for Bone Regeneration and Drug/Gene Delivery**

Due to their mechanical properties, both CNFs and CNTs have been investigated in order to improve the performance and the lifespan of scaffolds for bone regeneration.

In one of the first studies [50], carbon fibers of nanometer and micrometer scale have been integrated into a polycarbonate urethane (PCU) scaffold and cell adhesion of
osteoblasts, chondrocytes, fibroblasts and smooth muscle cells have been tested. As a result, osteoblasts adhered only on CNFs-PCU whereas all the other cells preferred larger-scale carbon fibers. Another important finding of this research was that chondrocytes, fibroblasts and smooth cell adhesion was inversely proportional to surface energy whereas a direct proportion was detected for osteoblasts. Such a material would be perfect for bone tissue regeneration as it could improve the integration of bone tissue in the implant, preventing the adhesion of every other cell apart from osteoblasts [51].

In another study [52], carbon fiber compacts were fabricated and fibers with diameters smaller or bigger than 100 nm have been used. Osteoblasts were then seeded on such compacts and their long terms cellular functions were compared. The results showed that cells produced more alkaline phosphatase (a bone formation marker) and deposited more extracellular calcium on the nanophase compacts compared to bigger scale compacts. This investigation not only proved CNFs biocompatibility but also their enhancing-cell-function properties [53].

Li et al. [54] fabricated composites from either MWCTNs and graphite where Myoblastic mouse cells (C2C12) were cultured. They found better cell attachment, proliferation and differentiation on MWCNT compared to graphite and proved that these results were mainly determined by a mechanism of increased protein adsorption on MWCNTs [54]. Moreover, the higher production of alkaline phosphatase, suggested that MWCNTs can induce C1C12 cells to differentiate into osteogenic cells more than graphite. As a consequence they defined MWCNTs as an "osteoproductive" material.
due not only to the elicited extracellular cell responses, as osteoconductive materials, but also to the intracellular responses [51].

The in vitro studies described above show how the applications of CNFs and CNTs in bone regeneration can lead to several improvements.

Another recently investigated application of CNTs and CNFs is in drugs and gene delivery in order to specifically target a group of cells to be treated via genes or medications. The internalization of CNTs and CNFs seems to take place following two types of different pathways. In the first one, the natural mechanism of endocytosis is involved whereas, in the second one, a non-dependent endocytosis process but an insertion and diffusion of CNTs or CNFs through the lipid bilayers of the membrane are the driving forces [51]. Functionalization of both CNFs and CNTs is very important in order to bind the drug or the genes needed to be released inside the cell as well as to favor the interactions between these materials and the cell membrane.

For instance Liu et al. [55] used SWCNTs functionalized with poly(ethylene glycol) (PEG) and conjugated with paclitaxel (PTX) (a widely used cancer chemotherapy drug) to kill tumor cells in mice.

Their results showed that as-functionalized SWCNTs not only did not cause any toxic effects, but also that they could stay in the blood for longer times than Taxol (the clinical drug formulation of PTX) and PTX coated with PEG and moreover, this prolonged circulation time, allowed a higher accumulation of the drug at tumor sites [51].

Another important property of CNTs that can be exploited in drug delivery is their strong adsorption in the near-infrared region (NIR). In fact, tumor treatment can be performed also using the hyperthermia treatment where heat, specifically released,
causes tumor cells death. SWCNTs can be effectively heat up in vivo using NIR light so that, once inside the tumor cells, they can kill them without affecting the healthy tissues in the surrounding where the optical absorption in the NIR is minimal [51]. Kam et al. [40] functionalized SWCNTs with folic acid (FA) through PEG chains and phospholipids (PL) to create SWCNT–PEG–FA conjugates. FA was chosen as it can bind specifically to folate receptor (FR) tumor markers, over-expressed in several cancer cell types. They discovered that these conjugates were able to enter inside the cancer cells but not in the normal cells and, once applied the NIR radiations, the tumor cells were effectively killed by the SWCNTs [40].

CNFs and CNTs have also been applied for gene delivery purposes. The main goal of this application is to make the cells able to produce specific therapeutic proteins by themselves without directly introducing drugs. Two main techniques can be used for this purpose: viral gene delivery, where genes are carried by a non-virulent virus into the cells, or non-viral delivery of genes to cells that uses means other than viruses (for instance injection) to transport genes inside the cells [51]. McKnight et al. [56] used vertically aligned CNFs (VACNFs) grown on flat substrates to deliver DNA to a large number of cells with the advantage of minimal cell membrane disruption during the injection (due to the small size of CNF tips). The cells normally proliferated and the expression of the delivered DNA, that coded for a green fluorescence protein (GFP), was clearly detected [56].

Moreover, amino-functionalized MWCNTs (NH2-MWCNTs) conjugated with plasmid DNA have been obtained by Gao et al. [57]. Such MWCNTs were able to deliver a GFP gene into cultured human umbilical vein endothelial cells.
One of the advantages about using CNTs for gene delivery is that from molecular
dynamic simulations it seems that DNA can actually bind spontaneously to the CNT
walls due to Van der Waals interactions and therefore without the need for other
functionalization [58].

**CNTs/CNFs for Neural Applications**

Due to their electrical and mechanical properties as well as to their nanotexture
that mimic neurons’ natural environment, CNTs and CNFs have been studied and
investigated for several different types of neural applications.

These nanomaterials could lead to optimal solutions for multiple problems
affecting traditional neural implantation and surgery. For instance, electrode-based
prosthesis made of silicon or other metal alloys are subjected to natural body’s
mechanisms of defense that cause encapsulation of the foreign material and therefore
an increase in electrical resistance between the probe and the tissue so that the
functionality and effectiveness of the electrical stimulation become impairing. Moreover
some of the biomaterials use for nerve conduits regeneration lack in mechanical and
electrical properties, preventing the desired and expected beneficial results [51].

Vertically aligned carbon nanofiber (VACNF) electrode array has been used by
Nguyen-Vu et al. [59]. They showed that an intimate neural–electrical interface between
PC12 cells and NFs can be obtained when the VACNFs were coated with electrical
conductive polypyrrole (PPy) polymers and with collagen extracellular matrix (ECM).
The strong 3D structure and the high electrical conductivity of the nanoelectrode have
been considered by the authors as the main reasons for such relevant results.
Other researchers have tested the same VACNFs-electrode, recording both spontaneous and induced neuroelectrical activity of organotypic hippocampal slice cultures. Potential advantages over traditional metal electrodes have been shown [60].

In order to solve the problem of encapsulation by glial scar tissue happening on electrodes implanted in the brain, a study [61] investigated how astrocytes behave on polycarbonate urethane (PCU) doped with CNFs. When the surface energy of CNFs was increased fewer astrocyte cells adhered on the composite material. Moreover, by increasing CNF weight ratios, the composite’s electrical resistivity was shown to decrease. These two important results show that CNFs could be very useful in order to fabricate electrodes with different conductivities at different parts and to reduce the adhesion of undesired cells [51].

Dubin et al. [62] fabricated, from SWCNTs, carbon nanotubes wires and cultured on them hippocampal neurons and PC12 cells. They were able to demonstrate the biocompatibility of these nanostructured materials shown that cells could grow, spread and attach on them. Huang et al. [63], similarly to Dubin et al., created a CNT rope substrate, from CVD-nanotubes, where neural stem cells (NSCs) in culture medium were stimulated and their response after stimulation was observed. The results showed that the electroconductive CNT rope substrate along with electrical stimulation can promote neurite elongation and boost effects on the differentiation of NSCs into mature neuronal cells [63].

Mattson and colleagues [64] showed for the first time that MWCNTs, coated with bioactive molecule 4-hydroxynonenal, support neural cell attachment and growth. In another study [65], glass coverslips coated with thin films of SWCNTs-
polyethyleneimine (PEI) copolymer allowed neurons to grow and extent as well as they did on only-PEI coated samples. Matsumoto et al. [66] proved that MWCNTs covalently bonded with neurotrophin can regulate and promote neurite outgrowth.

The high electrical conductivity of CNTs has been exploited by Lovat et al. [67] by using MWCNTs to improve electrical signal transfer on hippocampal neurons so that spontaneous firing activity recorded through single cell patch clamp was significantly increased on coverslips coated with unfunctionalized MWCNTs-film compared to uncoated glass coverslips.

Mazzatenta et al. [68] grew hippocampal cells on pure SWNT substrates and on control glass coverslips. Spontaneous electrical activity was recorded using patch clamp and applying current through the nanotube substrate they were able to induce action potentials in the cultured neurons. Moreover, after stimulation, postsynaptic currents were observed indicating neuron–neuron signaling [69].

Different studies have investigated the advantages of using CNTs on electrodes for either in vivo implants (i.e. cochlear prosthesis, deep-brain stimulation implants and neuro-motor prosthesis) or in vitro recording. Lowering the impedance and increasing the charge transfer compared to regular metal electrodes were the main results expected. According to Keefer et al. [70], tungsten and stainless steel wire electrodes coated with MWCNTs promote both the recording and electrical stimulating characteristics of neural electrodes compared to non-coated ones [51]. They tested both coated and bare metal electrodes in vivo on motor cortex of anesthetized rats and visual (V4) cortex of a trained monkey. Results showed reduced noise and increased sensitivity in the detection of spontaneous electrical neuronal activity on MWCNTs-
electrodes compared to control electrodes [69]. CNTs have been applied also on metallic electrochemical electrode arrays (multi-electrodes arrays, MEAs) for the in vitro recording of neurons in order to investigate biological mechanisms underlying neuronal systems. These devices for in vitro analysis of neurons, often termed “brain on a chip” (or neurochip), need to be manufactured in such a way that the actual interface between the chip and the cells is biocompatible and, at the same time, ensures effective electrical transmission. To optimize the multi-electrode array so that its capacitance is large but its projection small in order to allow high specificity, they can be coated with CNTs, exploiting their high capacitance and surface area [1]. Ben-Jacob and Hanein [1] realized CNTs-coated electrodes and placed mouse retinal whole mounts onto them with ganglion cells (the output cells of the retina) in direct contact with the electrodes. Spontaneous activity was recorded indicating the ability of these electrodes to record from intact neural tissue as well as to stimulate the same tissue. As a conclusion they showed that the CNT-electrodes have a cell-adhesive nature important for promoting neuronal proliferation and improving tissue–electrode interaction [1]. Chen et al. [71] worked as well on CNT-MAE in order to decrease their hydrophobicity. MWCNT were grown from nickel–titanium multilayered metal catalysts through thermal CVD and then treated with plasma. Such manufactured MEA was used to record neural signals of a lateral giant cell from an American crayfish. The results obtained showed improvements in electrode’s performance such that they would make feasible the separation of neural signals recorded and the recognition of their distinct shapes [71].

Neurons’ behavior on patterned networks of CNTs have been investigated by Gabay et al. [72] and by Sorkin et al. [73]. In the first study a pattern of iron nanoparticle
islands were imprinted on quartz substrates and, through CVD, CNTs were grown on the iron catalyst islands.

After 4 days culture, neurons accumulated on the CNTs islands with neuronal processes bridging them [72]. In the second investigation, iron nitrate catalyst was patterned on coverslips with a polydimethylsiloxane stencil and then CNTs were grown by chemical vapor deposition. Hippocampal neurons cultured on these substrates grew spontaneously into islands after 3 days seeding [73].

CNTs have also been used in stem cell investigation and important results have been obtained. Jan et al. [74] cultured mouse embryonic neural stem cells (NSCs) from the cortex onto LBL-SWCNTs-PEI thin films on glass coverslips and compared cells’ growth and attachment on these surfaces control coverslips coated with poly-L-ornithine (PLO). They showed that stem cells can be successfully differentiated into neurons, astrocytes, and oligodendrocytes on SWCNTs-PEI samples as they can do on PLO samples.

**Toxicity of CNTs and CNFs**

Despite all the important properties of CNTs and CNFs described above and of all the good results obtained from their applications in tissue regeneration and neural engineering, several studies have elucidate their possible toxicity. Lung toxicity, skin irritation and cytotoxicity are the three main areas of investigation.

Using intratracheal instillation techniques and pharyngeal aspiration on guinea pigs, researchers found large CNT agglomerations in the proximal alveolar regions of the lungs, as well as, fine fiber-type structures in distal alveolar regions [75]. In the majority of these studies were observed CNTs-dose-dependent inflammations, lung lesions and granuloma formation whenever either unpurified SWCTNs or unpurified...
MWCTNs have been tested [76][77]. Moreover, even functionalizing the CNTs with surfactant or sonication, do not seem to help as the agglomerative nature of CNT in aqueous solution is unavoidable inside the body and this has been pointed as one of the possible reasons for CNTs lung toxicity [75].

Skin irritation due to CNTs has not been directly proved whereas two investigations on human epidermal keratinocytes incubated with CNTs, unrefined SWCNTs [78] and purified MWCNTs [79] respectively, have been carried out. The results of both these studies showed loss in cell viability and morphological alterations to cellular structure in a time-dose dependent manner.

Cytotoxicity of CNTs has been investigated on several different types of cells: epidermal keratinocytes as described before, peritoneal and alveolar macrophages, embryonic kidney (HEK293) cells, PC12 and 3T3 fibroblasts. To evaluate the inflammatory potential of CNTs, Muller et al. [80] incubated peritoneal macrophages in media containing purified MWCNTs and purified “ground” MWCNT (the sample was ground in an oscillatory ball mill) at concentrations of 20, 50 and 100 μg/mL. They found that “ground” MWCNTs had a much higher capacity for inducing dose-dependent cytotoxicity (measured according to lactate dehydrogenase (LDH) release) and up-regulating TNF-α (a pro-inflammatory cytokine) expression compared to “un-ground” MWCNT. Cui et al. [81] cultured HEK293 cells in media containing concentrations of SWCNT ranging from 0.78 μg/mL to 200 μg/mL. Adhesion ability and protein secretion of the cells were tested coupled with genetic expression of cells cultured with SWCNT obtained through biochip analysis. Their results showed a decreasing in cell proliferation and adhesion ability following a dose- and time-dependent manner. Moreover, genes
involved in apoptosis were up-regulated, while genes associated with the G1 phase of the cell cycle (the major period of cell growth) and with adhesion were down-regulated [75]. Nimmagadda et al. [82] investigated viability and metabolic activity of 3T3 cells incubated with media containing varying concentrations of CNTs (0.001–1.0% (wt/vol)). In particular, three different SWNT preparations were analyzed: as purchased (AP-NT), purified (PUR-NT), and functionalized with glucosamine (GA-NT) [83]. They found that cell viability was inversely proportional to concentrations of SWNT and that was dependent on SWNT preparation. The same thing appeared to be true for cells metabolic activity. In another work from the same group [84], four different SWNT preparations (unrefined, refined, and SWNT with either albumin or human plasma adsorbed) were tested in a 3D environment using a red blood cell lysis model using PC12 and 3T3 cells. Purification was addressed as extremely important in order to reduce cytotoxicity as with purified CNTs the lysis of red blood cells (RBCs) was reduced from 11% to 0.7%. On one end, human plasma or albumin physisorbed were shown to improve hydrophilicity as well as RBC interactions but, on the other end, they caused a significant reduction in CNT-conductance.

From the studies here described, the possible toxic effects of CNTs have to be taken into account, even more in biomedical applications, in order to properly take advantage of their very good properties (mechanical, electrical etc.) with respect to cell viability and activity.
CHAPTER 3
MATERIALS AND METHODS

Experimental Design

All the experiments carried out can be divided into two main groups, one involving CNTs and the other involving CNFs.

The first set of experiments is supported by the idea that CNTs, once applied on electrode’s surface, reduce the impedance and allow a better signal transmission and recording. In particular, our lab has a long experience on micro electrode arrays (MEAs) for in vitro recording of neurons and the possibility to increase electrode’s capacitance is considered an important issue. In order to understand if such a CNT-surface modification can be considered biocompatible for neuronal cells we investigated how neuron’s morphology changes once they have been plated on these substrates. Moreover we wanted to investigate if fabricating two types of CNT-surface, one rougher than the other, would make any differences in cell viability and neurites development. In order to do that we used a layer by layer deposition technique, that will be explained later in details, as it an easy and repeatable way to obtain a controlled CNT-surface modification. Our hypothesis was that increasing the number of CNT layers (from 3 to 9) would increase the roughness of the surface and therefore improve cell attachment and development when comparing the neurons behavior on 9 LBL to 3LBL and to the control surface.

The second set of experiments was driven by the idea that a surface even rougher than the 9LBL surface, with the same carbon structure and electrical properties, could improve cell viability and neurites development. Therefore we used CNFs, already available to us, obtained from the carbonization of SU8 nanofibers and treated with
polyethyleneimine (PEI), a polymer solution commonly used in neural cell culture to improve cell adhesion. From preliminary results we found that using a standard concentration of 0.1% PEI on CNFs led to cell death. For that reason we tried three different types of PEI concentration (0.1%, 0.001%, 0.00001%) to understand if the shown PEI toxicity on CNFs would have been reduced diluting the polymer-solution.

We hypothesized that there might be an interaction of PEI adhesion to the tortuous surface of the nanofibers, raising the apparent concentration and leading to cell death. Hence we predicted that lower concentrations would lead to greater cell survival and growth, perhaps selectively on the nanofibers with greater convoluted structure. Moreover, in order to deeper investigate at which extent the nanostructure or the mainly carbon composition of the material can affect neuron’s growth, we also tested neuronal development on carbonized thin film of SU8 (CTF). In line with that, we seeded cells also on uncarbonized SU8 nanofibers (SU8NF) and uncarbonized SU8 thin film (SU8TF) to assess whether the nanostructured and uncarbonized material would enhance neuronal growth and, at the same time, if the SU8 material by itself, without any other modifications added, could improve neurite outgrowth.

**Cell Culture**

E18 cortices (Brainbits, LLC) were dissociated according to the vendor’s protocol. Specifically, the cortices have been digested in a sterile, 2mg/mL papain solution in Hibernate E (Brainbits, LLC) at 35°C for five minutes. Then the tissue clump has been removed from the papain solution and dispersed via trituration in the original shipping solution (Hibernate E/B27). The cell suspension has been centrifuged at 1000 rpm for 1 minute, the supernatant has been removed, and the cells resuspended in Neurobasal™/B27/GluMax™ media. A small sample of cell suspension (20 µL) has
been mixed with 20 µL of Trypan Blue (Hy Clone®, Utah) and the cells have been counted on a hemocytometer (typically around 4-6x10^6 cells/mL). The cells have been plated at 150 cells/mm² on both the CNT and CNF samples. The cultures were kept in an incubator at 37°C and 5% CO₂ for 3 and 7 days respectively.

**Substrates**

**CNT Substrates**

The CNT coating was performed using a CNT solution and PEI solution.

The CNT solution was prepared using “HD plasma Multiwall Carbon nanotubes-COOH rich” purchased from “Cheaptubes”. The vendor specifies that they have been made from graphite through a CCVD process. They have been sterilized and functionalized with carboxyl group using a so called “Split Plasma” technique and no acids have been used. These are their properties:

- Outer diameter: 13-18 nm
- Length: 3-30 µm
- Purity > 99wt%
- Functional content: 7,00wt% +/- 1,5%

The CNT solution was prepared dispersing COOH-MWNTs at 0.2mg/mL in dimethyl formamide (DMF; 200-679-5, Fisher) by utilizing a pulsing (2s on, 1s off) ultrasonicator probe at 10% power for 40 min. Pulse mode was chosen in order to avoid precipitation of nanotubes out of solution due to thermal heating. The solution was then centrifuged at 3000 rpm for five minutes and the supernatant was extracted to prevent any unsolubilized clumps of COOH-MWNTs from compromising the uniformity of the films.
The PEI solution was prepared by diluting 50% (w/v) PEI (P3143, Sigma Aldrich) to 0.1% (w/v) in deionized (DI) water. The PEI used has a branched structure as shown in Figure 3-1.

The CNT samples were prepared using glass coverslips (22x22 mm). At first we treated them using a plasma system (MARCH PLASMOD, GCM-200) with oxygen plasma for 30 seconds. Then the CNT solution was deposited through a layer by layer (LBL) deposition method. In practice two separate tubes were filled either with CNT solution or PEI solution and each coverslip was immersed in each tube for 10 minutes. We began the sample preparation immersing it firstly in the PEI solution. After 10 minutes, the sample was taken out from the tube using tweezers, washed spraying on both sides of the samples DI water, dried using a nitrogen air gun and immersed in the CNT solution. After 10 minutes the sample was rinsed again with DI water, dried and immersed in the PEI solution. The same process was repeated 3 times for half of the samples (3LBL samples) and 9 times for the other half (9LBL samples).

In order to have both the LBL surface and the control surface on the same sample we filled the tube with CNT solution in such a way that only half of the sample was dipped into it. As a consequence after the LBL procedure half of each sample was covered by layers of both CNT and PEI whereas the other half was just coated with PEI layers (Figure 3-2). Sterilization was performed immersing the samples in 70% ethanol for 10 minutes.

**CNF, CTF, SU8NF and SU8TF Substrates**

The CNFs samples have been prepared by P. Jao and colleagues [48] from the Electrical and Computing Engineering Department of the University of Florida. In short, they used a photopatternable epoxy SU-8 2025 (Microchem Inc.) (Figure 3-3) diluted in
cyclopentanone (Sigma Aldrich Inc.) at a concentration of 60.87 wt%. The polymer solution was loaded without the introduction of bubbles into a syringe with a 21 gauge hypodermic needle (CML Supply, LLC). The solution was pumped with a steady flow of 0.5ml/hr, maintained using a syringe pump (NE-1000, New Era Pump Systems, Inc., USA). A voltage in the range of 10kV to 15kV was applied to the needle using a high voltage supply (DEL HVPS MOD 603 30KV POS, Spellman High Voltage Electronic Corp., USA). The electrospun SU8 nanofibers were collected on a silicon wafer (p type, University Wafers Inc., USA). Lithography patterning of the SU8 nanofibers was then performed using the standard 365nm i-line UV mask aligner (MA6, Karl Suss Inc., USA) following the standard processing conditions.

To remove any trapped solvent the sample was baked in an oven. Carbonization of the SU8 nanofibers was then performed in a tube furnace (Lindberg tube furnace, Fisher Scientific, USA) with an inert atmosphere of forming gas (H2:N2 5%:95%) at 1000°C [48]. SU8TF sample were manufactured depositing SU8 as a thin film on silicon substrates (Figure 3-4). Carbonization at 1000°C was followed to obtain the CTF-substrates.

All the samples (CNF, CTF, SU8NF, SU8TF) were at first plasma treated for 30 seconds. After that, some of them were coated with PEI solution at a concentration of 0.1%, some others at a concentration of 0.001%, the remainder at a concentration of 0.00001% with few of them that did not undergo PEI treatment at all. Once PEI was applied on the samples, it was left seeding for 30 minutes and then 3 cycles of rinsing with DI water were performed. After drying the samples, dissociated neurons (above
described) were plated on them with a density of 150 cells/mm² and the cells were let grow for 3 days (3 DIV).

**Imaging**

**Fluorescence Microscopy**

The cells were stained using LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Cat. No. L-3224) composed of acetomethoxy derivate of calcein (Calcein AM) and ethidium homodimer-1 (EthD-1). Calcein AM is a polyanionic dye containing an acetomethoxy group that, once the molecule has crossed the cell membrane, is removed by intracellular esterases and a strong green fluorescence is produced. The fluorescence has an excitation/emission wavelength of 495 nm/515 nm. EthD-1 is a membrane-impermeable fluorescent dye which binds to DNA and can enter the cell only when cells die and the plasma membranes becomes disrupted. It is weakly fluorescent until bound to DNA. At that point it produces a bright red fluorescence in dead cells with an excitation/emission wavelength of 495 nm/635 nm.

According to vendor’s protocol the reagents were prepared adding 20 µL of 2 mM EthD-1 stock solution to 10 mL of sterile, tissue culture-grade D-PBS to which 5 µL of the 4-mM calcein AM stock solution was added. The ~2 µM calcein AM and ~4 µM EthD-1 solution prepared were added to the plated cells and incubated for 30 minutes. The samples were then analyzed using an inverted light microscope (IX51, Olympus, USA) and pictures were taken using a camera integrated in the microscopy system and SPOT Basic™ image capture software (version 4.6).

**AFM**

The roughness of the CNT and CNF surfaces was evaluated using an atomic force microscope (AFM), Veeco Dimension 3100 SPM and AFM System. The scan was
run in tapping mode that operates by scanning a tip attached to the end of an oscillating cantilever across the sample surface. The scan size was set at 10 µm and the frequency at 0.5 Hz. The AFM images were analyzed using Gwyddion software [85] (free scanning probe microscopy data analysis software) and average roughness $R_a$ was derived.

**SEM**

The samples were firstly coated using Gold palladium (AU-PD) with Denton DeskV Sputter coater. SEM analysis was then performed using Hitachi S-4000 FE-SEM. The pictures were taken with the samples positioned on the horizontal plane as well as tilted of 45°.

**Image Analysis: NeuriteQuant**

NeuriteQuant is an open source ImageJ plugin that allows quantitative image analysis of neuronal morphology. In the work by Dehmelt et al. [86] a detailed description of the software and results obtained from its application in image analysis of high density differentiated P19 stem cells and Primary Hippocampal Neurons are presented. Here we will briefly outline the image processing algorithm of the system.

The method for morphological analysis used in NeuriteQuant is based on the public domain Greyscale Morphology filter by Dimiter Prodanov that performs the basic operations of erosion, dilation, opening and closing with several types of structuring elements. Therefore cell morphology detection is based on the enhancement of small elongated (neurite-like) structures or circular (cell body-like) structures and not on signal intensities so that both neurites containing abundant signal and those scarcely detectable above background can be detected [86].
The graph in Figure 3-7 points out all the phases of the image analysis process needed to obtain the primary measurements: neurites, cell bodies, endpoints and attachment points. The plugins works only with 8-bit images so, before beginning the analysis, we need to convert the RGB image into 8-bit image using ImageJ (Figure 3-5).

At first we have the original 8-bit image to which an opening filter is applied in order to get only the cell bodies (1). The image is then transformed into a black and white image through binarization (2) and the cell bodies are obtained. Binarization is applied according to a specific threshold that the user can manually set using ImageJ as shown on Figure 3-6.

The cell body non-binarized image is subtracted from the original image in order to keep just the fibrous structure of the neurites (3). Binarization is performed on this image (4) and size filtering is applied to remove debris or imaging artifacts (5). This special filter, which allows filtering objects enclosed by larger objects, is not originally included in the ImageJ package so that it must be integrated in the software together with NeuriteQuant. The cleaned up picture is then skeletonized into one pixel wide structure (6). After skeletonization, some of the skeleton components can be found also in areas that should be occupied only by cell bodies. In order to identify such components, the skeletonized image is superimposed to the binarized cell bodies image (7). The obtained image is then subtracted from the skeletonized one (8) and the image with only neurites is obtained. The endpoints are detected eroding the single terminal pixel of the skeletonized fiber structure (9). Subtracting that image from the original skeletonized one, a rough endpoints image is obtained (10). The same procedure applied for the neurites in order to erase those components that were found to be on the
cell body areas, is applied here. The rough endpoints image is superimposed to the cell bodies image and the endpoints found on these areas are erased (11) (12). The endpoints image is obtained. Dilation is applied to the cell bodies image in order to include the area around the cell bodies where the attachment points are (13). This image is superimposed to the skeletonized image and the components of the skeleton that are found inside the dilated cell bodies are detected (14). Erosion is applied so that the single terminal pixels are cut off (15). Subtracting this image from the non-eroded one the rough attachment points image is obtained (16). Finally the non-dilated cell bodies image is superimposed on the rough attachment points image and the attachment points that are found to be inside the cell body areas are erased (17) [86]. An example of the image that we will obtain at the end of the analysis performed by NeuriteQuant is shown in Figure 3-8.

From the image analysis performed following the described algorithm, several parameters are reported by NeuriteQuant: total neurite length (measuring the total length of the skeletonized structure), total neuronal cell body area, total number of cell bodies, number of attachment points (number of neurites), and number of neurite endpoints. These are defined as primary measurements from which additional neuromorphological features are derived. For example the total number of branch points is obtained as the difference between neurite endpoints and neurite attachment points (Figure 3-9). Average cell body area (dividing the total neuronal cell body area by the total number of cell bodies), average neurite length per neuron (dividing the total neurite length by the total number of cell bodies), average length of individual neurites (dividing the total neurite length by the number of attachment points), branches per neurite
(dividing the number of branch points by the number of attachment points) and branches per cell (dividing the number of branch points by the number cell bodies) are also provided by NeuriteQuant.

The unit of measure used by NeuriteQuant is pixel. In order to convert it into meter scale, using an hemocytometer picture, we measured that, for a picture of 10x magnification (the magnification applied to all the pictures taken), 0.25 mm is equal to 340 pixels. This conversion factor was applied to all the measurements involving length and area.

In regards to the CNF, CTF, SU8NF and SU8TF pictures, a more elaborated process preceded and followed the analysis. In fact, in order to get separate measurements for the strips and the silicon present in the same picture, each image was modified in such a way that two different pictures could be obtained, one with only the fiber and one with only the silicon-background (Figure 3-10).

Once NeuriteQuant analysis (for the CNF and CTF samples) and cell count (for the SU8NF and SU8TF samples) have been performed, the length and area measurements obtained were converted into meter scale using the same conversion factor explained before and were also divided by each measured area (nanofibers or silicon area) so that all the measurements were only related to the specific area of the sample that underwent the analysis. In each experiment, two or more pictures of the same sample have been taken so that the values, obtained from NeuriteQuant analysis, for each picture of the same type of sample have been averaged and the standard deviation calculated.
Among all the parameters offered by NeuriteQuant, we focused our attention on the percentage of live cells over the total number of cells (live and dead cells), the cell body average size, the neurite length per neuron, the number of neurites per neuron, the length of individual neurite, the number of branches per neurite and the number of branches per cell.

**Statistical Analysis**

At first we calculated the average and the standard deviation of all the values obtained for each one of the parameters. In order to find which differences were significant, we performed t-test, ANOVA and Tukey test using BrightStat, a free online application for statistical analysis. The difference between two values was defined as significant, and indicated in the graphs with a star, when the p value obtained was equal or smaller to 0.05.

![Branched PEI molecule](image)

Figure 3-1. Branched PEI molecule.
Figure 3-2. Drawing of a LBL sample. On the left is shown the glass side of the sample treated with PEI 0.1% while on the right is shown the CNT side of the sample that could be either CNT(3LBL) or CNT(9LBL).

Figure 3-3. Chemical structure of the Bisphenol A Novolak epoxy oligomer contained in SU-8 formulations [87].

Figure 3-4. Drawing of a CNF/CTF/SU8NF/SU8TF sample. On the upper left corner there are the stripes which can either be made of CNFs or SU8NF or just be CTF or SU8TF. The background of the stripes is made of silicon as the surrounding area.
Figure 3-5. Conversion of the image from RGB type to 8-bit type using ImageJ. A) RGB image. B) 8-bit image.
Figure 3-6. Example of threshold setting with ImageJ. A) We set a threshold for neurite detection trying to be as much inclusive as possible so that all the neurites are picked up and red colored. B) We set a threshold for cell bodies detection so that only the cell bodies are white colored.
Figure 3-7. Schematic representation of the algorithm used by NeuriteQuant for image analysis [86].
Figure 3-8. Image obtained at the end of NeuriteQuant analysis where in purple are indicated the endpoints, in white the attachment points, in yellow the cell bodies and in blue the neurites.
Figure 3-9. Primary and secondary measurements that can be obtained from NeuriteQuant.
Figure 3-10. Example of image modifications applied to CNF, CTF, SU8NF, SU8TF pictures before NeuriteQuant analysis. A) Original picture of 0.001% PEI CNF. B) Modification of the original picture where only neurons on silicon are visible. C) Modification of the original picture where only neurons on the stripes are visible.
CHAPTER 4
RESULTS AND DISCUSSION

CNT Samples

AFM and SEM Analysis

From both AFM and SEM pictures we were able to define the difference between CNT(3LBL) and CNT(9LBL). In terms of roughness, the CNT(9LBL) appeared to be only slightly rougher compared to CNT(3LBL).

In particular we calculate the roughness average (Ra) as the average of five Ra measurements obtained from different lines drawn on each AFM pictures (Figure 4-1) using Gwyddion software. The Ra for the CNT(3LBL) sample was equal to 12 nm whereas for the CNT(9LBL) was equal to 17 nm. The SEM images shown below (Figure 4-2) confirm the variations in roughness assessed through AFM measurements.

NeuriteQuant Analysis

Using NeuriteQuant we analyzed the images obtained after fluorescent staining. The staining was performed, on two separate cultures, after 3 days and after 7 days.

On Figure 4-3 are shown the original pictures of the neurons grown on CNT(3LBL) and CNT(9LBL) at 3 DIV and 7 DIV. On Figure 4-4 are presented the images of neurons grown on PEI(3LBL) and PEI(9LBL) at 3 DIV and 7 DIV. The same images are presented after performing NeuriteQuant analysis on Figure 4-5 and 4-6.

In Figure 4-7 the values obtained for the percentage of live neurons and the cell body average size are shown.

In relation to the percentage of live cells we can see that the values oscillate between 45 and 60 per cent on both CNT side and control side (PEI). In particular, at 3 DIV cell survival is higher on PEI(3LBL) in comparison to PEI(9LBL) whereas cell
viability is in the same range on both CNT(3LBL) and CNT(9LBL). At 7 DIV the percentage of live cells decreases of 10% on PEI(3LBL) and CNT(9LBL) whereas it remained stable on PEI(9LBL).

About the cell average size we can see that it is larger on the control side in comparison to the CNT side (for CNT(3LBL)-PEI(3LBL) p=0.05 at 3DIV and p=0.024 at 7DIV, for CNT(9LBL)-PEI(9LBL) p=0.007 at 7DIV and p=0.001 at 3DIV). At three days the average cell body area is approximately equal to 220 µm² on PEI(3LBL) and to 300 µm² on PEI(9LBL). The average size of neuron’s cell bodies on both CNT(3LBL) and CNT(9LBL) is attested as equal to 170 µm². At 7 DIV we can see the same trend proportionally increased for each type of sample even if in this case we found a significant difference between the cell average size on CNT(3LBL) and CNT(9LBL) (p=0.013).

Figure 4-8 shows the results obtained for the neurite analysis. In the first panel the total neurite length per each neuron is presented. At 3 DIV the length is assessed equal to 450 µm for both CNT(3LBL) and CNT(9LBL) and slightly longer (550 µm) for both PEI(3LBL) and PEI(9LBL) (p=0.008 between CNT(9LBL) and PEI(9LBL)). At 7 DIV the neurite length increases of three folds on the CNT side and of four folds on the PEI side reaching the value of 1750 µm and 2000 µm respectively but these values were not found to be significant. At 3 DIV, the neurites per cell are equal to 5 on both CNT sides and on PEI(3LBL) with an extra one for the PEI(9LBL) (p=0.052 between CNT(9LBL) and PEI(9LBL)).

Over time, other extra 4 neurites grew on both PEI sides whereas only 2 on CNT sides and all the values were found to be significant (p=0.008 between CNT(3LBL) and
PEI(3LBL) and p=0.004 between CNT(9LBL) and PEI(9LBL)). The average length of each individual neurite is directly related to the number of neurites per cell and the neurite length per neuron described above. We can see that the neurite average length is approximately equal to 100 µm at 3DIV on almost all the samples whereas it increases up to 250-300 µm at 7 DIV.

Figure 4-9 represents the values obtained for the branches. According to NeuriteQuant definition, “branch” is every ramification a neurite undergoes. The parameter “branches per neurite” estimates how many branches developed from each neurite. At 3 DIV the number of branches is of 4-5 on both PEI and of 2-3 on both CNT sides (here we found a significant difference between CNT(3LBL) and CNT(9LBL) with a p-value equal to 0.03 and between CNT(9LBL) and PEI(9LBL) with a p-value equal to 0.009). At 7 DIV the number of branches increases of about 4 times on the CNT sides and of less than 2 times on PEI sides (CNT(3LBL)-PEI(3LBL) p=0.018, CNT(9LBL)-PEI(9LBL) p=0.017). The same thing can be seen for the number of branches per cell. At 3 DIV on both CNT sides less than 15 branches per neuron are counted and on the PEI sides almost 25 (significant differences were shown between CNT(3LBL) and CNT(9LBL) (p=0.026) and between CNT(9LBL) and PEI(9LBL) (p=0.001)). At 7 DIV, 60-70 branches are observed on the neurons on both the CNT sides and 50-70 branches are counted for the PEI(3LBL) and PEI(9LBL), respectively, even if from the t-test we found that these values were not significantly different.

Discussion

From the results shown and described above we can derive few interesting findings. At first, the fact that the percentage of live cells is only slightly different among CNT samples and PEI samples either at 3 DIV or 7 DIV tells us that the biocompatibility
of such modified surfaces is preserved. Moreover we have that the cell body average
size was found to be larger on PEI modified surfaces compared to CNT modified
surfaces. This can be explained referring to the studies carried out by Hu and
colleagues [88] where they showed that cell body area and neurite length are likely to
increase in size when the neurons are cultured on positively charged surfaces.
Therefore what we can hypothesize is that in the LBL samples the positive surface
charged given by the PEI is actually reduced by the presence of the CNTs negatively
charged so that the cell bodies are much smaller. If we look at the neurites and
branches results, we can see the same situation that only in few cases the difference
between the values for the CNT and on PEI surfaces were found to be significant. The
reason for that can be linked to the idea proposed by Hu et al. [65] that the surface
charge does not affect in the same way the cell body and the dendrites so that neurite
branching is defined as the least sensitive to the reduction of the substrate’s positive
surface charge. Another possible reason for the increased number of branches at 7 DIV
could be that the MWCNTs, with a diameter of 13-18 nm and a length of 3-30 µm, could
actually work as topographic cues for the development of distal braches that must have
a smaller diameter and a shorter length compared to proximal branches (neurites) that
instead do not seem to be affected by the presence of the CNTs.

In conclusion we want to highlight the fact that only in two cases we found a
significant difference between the CNT(3LBL) and the CNT(9LBL) samples among all
the parameters taken into account. This is an unexpected result as one of our starting
hypotheses was that, increasing the number of layers, the surface roughness would
have increased and therefore it would have enhanced neurite outgrowth. A possible
explanation could be that, even if the roughness actually increased with a higher
number of layers, this increment was not big enough to affect cell morphology.

**CNF, CTF, SU8NF, SU8TF Samples**

**AFM and SEM analysis**

AFM measurements were performed on the CNF, CTF, SU8NF and SU8TF
samples (Figure 4-10). On a scale of 10 µm the average roughness value for CNF
samples was measured to be equal to 160 nm, for the CTF to 0.26 nm, for the SU8NF
to 83 nm and for the SU8TF to 0.72 nm. In Figure 4-11 two SEM pictures of SU8NF and
CNF samples are shown. After the carbonization treatment the fibers shrink so that their
diameter decreases.

Figure 4-12 shows some pictures of the four types of samples (SU8TF, SU8NF,
CTF, CNF). The height of the stripes was measured with the following results: 6.25 ±
0.95 µm for the SU8NF stripes, 6.85 ± 0.00 µm for the SU8TF stripes, 7.64 ± 0.08 µm
for the CTF stripes and 5.24 ± 0.23 µm for the CNF stripes.

**NeuriteQuant Analysis**

In Figures 4-13 - 4-16 are presented some pictures of the neurons, at 3 DIV,
plated on the 4 different types of sample under the 4 different types of modification.

As explained before in the NeuriteQuant section, neurite analysis through this
software could only be performed on CNT and CNF samples. Therefore only cell
counting was performed on the SU8NF and SU8TF samples.

In order to compare the cell viability on the 4 different type of samples at the 4
different conditions already described (only plasma treatment, 0.1% PEI, 0.001%PEI
and 0.00001% PEI) a cumulative chart is presented in Table 4.4 showing the
percentage of live neurons over the total number of cells (live cells and dead cells) at 3
DIV. Generally speaking we can state that, when comparing the percentage of live cells on the stripes to the percentage of live cells on the silicon-background, similar values can be found when the samples have been only plasma treated or coated with 0.001% PEI whereas, at a PEI concentration of 0.1%, the cells prefer the silicon-background to the stripes (especially CNF- and SU8NF- stripes) even if these values were found not to be significantly different after Tukey analysis. Moreover we can see that at 0.00001% PEI outstanding cell viability can be seen on the stripes if compared to the background and Tukey test revealed significant the difference between the percentage of live cells on the CNF stripes and background (p=0.003), the SU8NF stripes and the background (p<0.001) and the SU8TF stripes and the background (p<0.001).

If we focus our attention only on the nanofiber-stripes of Figure 4-17 we can see that the percentage of live cells is the same for both CNF and SU8NF either only plasma treated or PEI treated. The only exception is at the lowest PEI concentration where the percentage is higher for CNF-stripes compared to SU8-stripes but this difference was not found to be significant. If we consider only the thin film-stripes, we can see that generally the percentage is higher on SU8TF compared to CTF but the situation is reversed at the highest PEI concentration. Comparing the carbonized stripes, the cell viability is higher on the CNFs excluding the case of PEI concentration equal to 0.1% where no cells have been found alive on the CNFs instead almost 50% of all the neurons plated on the CTF were still living. On the other end, if we compare the uncarbonized stripes (SU8NF, SU8TF) we can see that the percentages are almost the same for every surface treatment performed.
Focusing on the background results of Figure 4-17 we can state that good cell viability is shown on silicon either only plasma treated or 0.001% PEI treated. Instead a low percentage of live cells was detected when the silicon was treated with 0.1% PEI and 0.00001% PEI. Low cell viability on the background treated with 0.1% PEI was totally unexpected as this PEI concentration is commonly used in the literature for neuronal cell culture on silicon. At the same time we noticed that on the samples treated with 0.1% PEI, if there were no living cells on the silicon-background we found living cells on the silicon-surrounding area far away from the stripes. That means that for some reason that we cannot totally explained the cells did not like either the stripes or the background when treated with 0.1% PEI (with the exception for CTF samples) but survived on the surrounding area treated as well with 0.1% PEI.

In Figure 4-19 the bar chart shows the cell body average size of neurons grown on CNF and CTF at 3 DIV. We can see that the size of the cell bodies on the CNF stripes is bigger compared to the CTF stripes with the exception for the samples that underwent only plasma treatment where the cell body appeared to be bigger and the treatment with 0.1% PEI where all the cells died on the CNF stripes but preserved a good shape on the CTF stripes (p=0.001 between CNF and CTF at 0.1% PEI, p=0.028 between CNF and CTF at 0.001% PEI and p=0.002 between CNF and CTF at 0.0001% PEI).

If we compared the stripes and the background we can see that no significant differences have been detected with t-test analysis, only the cell average size of neurons on CNF treated with 0.001% PEI appeared to be smaller than the cell body size of neurons on the background (p=0.014).
The data referred to neurite analysis are shown on Figure 4-20. Here we can see that for all the three parameters taken into account (neurite length per cell per area, neurites per cell per area, average length of individual neurite) the neurons cultured on CTF stripes performed much better compared to neurons cultured on CNF stripes. From the t-test analysis we have that significant differences were found between the CNF and the CTF when plasma treated (p=0.004), 0.001%PEI treated (p=0.001) and 0.00001%PEI treated (p=0.004) for the neurite length, at 0.1%PEI treatment (p=0.001) and 0.00001%PEI treatment (p=0.033) for the neurites per cell and at 0.1%PEI (p=0.001) and 0.00001%PEI (p=0.011).

If we look at the background, from the t-test analysis, we found that when treated with 0.1%PEI, 0.001%PEI and 0.00001%PEI we have a significant difference among the neurite length (p=0.004 at 0.1%PEI and p=0.007 at 0.00001%PEI), the number of neurites per cell (p=0.001 at 0.1%PEI, p=0.025 at 0.001%PEI and p=0.005 at 0.00001%PEI) and the average length of individual neurites (p=0.003 at 0.1%PEI and p=0.007 at 0.00001%PEI) on the CTF-stripes compared to the background.

The number of branches per neurite and branches per cell for the CNF and CTF samples are shown in Figure 4-21. As seen for the neurite parameters, the neurons on CTF stripes performed better compared to neurons grown on CNF stripes with a higher number of branches per cell and branches per neurite under every type of treatment performed on the sample. The values of the branches per cell have found to be significant comparing CNF and CTF on samples treated with plasma (p=0.003), 0.1%PEI (p=0.001) and 0.00001%PEI (p=0.004). If we look at the background results, we can see a significant difference between CNF and background only if we compare
the branches per neurite after only plasma treatment (p=0.021). Instead significant
differences have been noticed between CTF and background in the values of the
branches per cell after plasma treatment (p=0.008), 0.1% PEI (p=0.005) and 0.00001%
PEI (p=0.003) and branches per neurite after 0.1% PEI (p=0.005) and after 0.00001%
PEI (p=0.003).

Discussion

In regard to the results described above about the percentage of live cells,
several comments need to be added. At first we must highlight the very high cell
viability, between 70% and 90%, observed on all the stripes with the exception of the
CTF-stripes where still a standard value is reached. Moreover, the fact that such
percentages were not observed on the background-silicon, considered the control
surface, is even more surprising.

The fact that neuron’s viability was almost the same on SU8NFs and SU8TF lets
us think that such a material, independently from the surface roughness, can have
important biocompatible properties at least in relation to neuronal cell culture. On the
other end, the fact that in general the difference between cell viability on CNF-stripes
and CTF-stripes was not find to be significant leads to the hypothesis that a carbon
structure by itself supports neuronal growth, with or without a specific degree of
roughness.

The results obtained in regard to the different PEI concentration confirm that
some specific interactions between PEI, at a concentration of 0.1%, and the stripes
prevent cell viability. The only exception to this phenomenon can be seen on the CTF
stripes where good cell viability was observed. As specified above, CTF stripes are
almost flat surfaces as SU8TF stripes whereas the surface of CNFs and SU8NFs
stripes is pretty much rough. Our initial hypothesis that could justify cell death was linked with the idea that the tortuous structure of the nanofibers-mat would somehow trapped PEI molecules so that the apparent concentration of PEI would increase reaching toxic levels that would prevent cell survival. In fact, it has been shown that PEI at high concentration can actually be toxic for cells due to the strong positive charge of the polycation which can lead to strong interactions of PEI with cell surfaces and consequently their damage [89].

If our initial hypothesis can explain cell death on CNF and SU8NF stripes at 0.1% PEI (due to their surface roughness) as well as the increasing cell viability with a decreasing in PEI concentration, it cannot explain why very low cell viability was also observed on SU8TF and on the silicon-background of all the stripes (exception for the background of the CTF stripes). In fact 0.1% PEI is the most used concentration for neuronal cell culture on glass and silicon that should enhance cell attachment and development and should not cause cell death. What we can hypothesize is that PEI can somehow interact with the SU-8 material, independently form the flat or the 3D structure given to it, causing cell death. Instead, for the carbonized stripes, we have that 0.1% PEI can become toxic only if the material has a 3D structure whereas it does not affect cell viability.

Very low cell viability has also been observed on the silicon-background when treated with 0.00001% PEI. In this case, due to the very high percentage of live cells on the stripes, we can hypothesize that the neurons actually migrated from the background to the NF. The pictures shown in Figure 4-13 validate this hypothesis even more as a lot of cells are actually distributed along the edges of the stripes.
Finally a general overview of the results obtained for CTF and CNF lets us think that neurons prefer much more the CTF stripes over the CNF stripes in relation to every parameter considered, with the exception for the cell average size. This outcome is actually very difficult to explain and does not satisfy the hypothesis that, keeping the same chemical composition of the surface but increasing its roughness, neurite outgrowth and branching would increase. For sure it tells us that neurons like carbon and that they also preferred it to the silicon background but in order to understand the very reason for that more analysis would have to be performed on the samples.

**Comparison Between CNT and CNF Samples**

We want to conclude this chapter comparing the results, already shown above, obtained from NeuriteQuant analysis of neurons cultured on CNT (both 3LBL and 9LBL) and CNF (only plasma treated or 0.1% PEI treated or 0.001% treated or 0.00001% PEI treated) samples at 3 DIV. From Figure 4-22 we can see that the percentage of live cells is generally greater on CNF (preferably only-plasma treated or treated with the lowest concentration of PEI) where a peak of almost 90% is reached. The cell body average size instead is pretty similar among the neurons cultured on CNF and CNT.

If we look at Figure 4-23, a bigger neurite length per cells and a higher number of neurite per cell are measured for neurons grown on CNF samples compared to CNT samples even if, from Turkey test, we found that the values obtained for the CNT(3LBL) were significantly different only compared to the CNF treated with 0.001% PEI instead for the CNT(9LBL) the values were significantly different to the values obtained for the CNF either treated with only plasma or 0.1% PEI or 0.00001% PEI. Again neurons are more likely to have a bigger number of neurites as well as longer neurite length when the CNF are treated only with plasma or with 0.00001%PEI.
The same description can be repeated for the charts shown in Figure 4-24 where the values for neurons cultured on CNF treated with plasma or a low PEI concentration are higher compared to all the other cases shown (i.e. CNT(3LBL), CNT(9LBL) and CNF treated with 0.1% PEI or 0.001% PEI).

In Figure 4-25 are presented the pictures of CNT(3LBL), CNT(9LBL), CNF plasma treated and CNF treated with 0.00001% PEI after been analyzed with NeuriteQuant. The increased number of neurites and branches when moving from neurons on CNT to neurons on CNF can be easily detected. At the same time we can see that the cell body average size is actually comparable among the four pictures.

We can conclude that in this case the theory, according to which a rougher carbon-surface (CNF-160 nm, CNT-17 nm) improves neurite outgrowth and branching, is totally satisfied. We should also add that if the increase in roughness affects all the neurites’ and branches’ parameters it does not seem to affect the cell body average size. A possible argument can be that this roughness is still too small to affect the cell body development that might need bigger topographical cues in order to increase in size.
Figure 4-1. AFM pictures of CNT(3LBL) and CNT(9LBL) samples. A) AFM picture of CNT(3LBL) sample. B) 3D representation of CNT(3LBL) sample’s surface. C) AFM picture of CNT(9LBL) sample. D) 3D representation of CNT(9LBL) sample’s surface.
Figure 4-2. SEM images of CNT(3LBL) and CNT(9LBL) samples. Images of CNT(3LBL) sample horizontally positioned (A) an tilted of 45° (C). Images of CNT(9LBL) sample horizontally positioned (B) and tilted of 45° (D).
Figure 4-3. Original pictures of neurons on CNT(3LBL) and CNT(9LBL) after 3 DIV and 7 DIV. A) CNT(3LBL) at 3 DIV. B) CNT(3LBL) at 7 DIV. C) CNT(9LBL) at 3 DIV. D) CNT(9LBL) at 7 DIV.
Figure 4-4. Original pictures of neurons grown on PEI(3LBL) and PEI(9LBL) at 3 DIV and 7 DIV. A) PEI(3LBL) at 3 DIV. B) PEI(3LBL) at 7 DIV. C) PEI(9LBL) at 3 DIV. D) PEI(9LBL) at 7 DIV.
Figure 4-5. Images of neurons cultured on CNT(3LBL) and CNT(3LBL) at 3 DIV and 7 DIV analyzed through NeuriteQuant. A) CNT(3LBL) at 3 DIV. B) CNT(3LBL) at 7 DIV. C) CNT(9LBL) at 3 DIV. D) CNT(9LBL) at 7 DIV.
Figure 4-6. Images of neurons cultured on PEI(3LBL) and PEI(3LBL) at 3 DIV and 7 DIV analyzed through NeuriteQuant. A) PEI(3LBL) at 3 DIV. B) PEI(3LBL) at 7 DIV. C) PEI(9LBL) at 3 DIV. C) PEI(9LBL) at 7 DIV.
Figure 4-7. Charts showing the values obtained from NeuriteQuant analysis for the percentage of live cells and the neurons’ cell body average size. The stars indicate which values are significantly different according to t-test analysis.
Figure 4-8. Charts showing the values obtained from NeuriteQuant for the neurite length referred to each neuron, the number of neurites per neuron and the average length of each neurite detected. The stars indicate which values are significantly different according to t-test analysis.
Figure 4-9. Charts showing the values obtained from NeuriteQuant analysis for the number of branches per each neurite and the number of branches for each neuron. The stars indicate which values are significantly different according to t-test analysis.
Figure 4-10. AFM pictures of CNF, CTF, SU8NF and SU8TF samples. A) CNF. B) SU8NF. C) CTF. D) SU8TF.

Figure 4-11. SEM pictures of SU8NF and CNF samples. A) Original SU8NF. B) Carbonized SU8NF (CNF).
Figure 4-12. Pictures of the stripes and the background of the four types of samples used. A) SU8TF. B) SU8NF. C) CTF. D) CNF.
Figure 4-13. Fluorescence Microscopy pictures of neurons on CNF, CTF, SU8NF, SU8TF samples only-plasma treated. A) CNF sample. B) CTF sample. C) SU8NF sample. D) SU8TF sample.
Figure 4-14. Fluorescence Microscopy images of neurons on CNF, CTF, SU8NF, SU8TF samples treated with 0.1% PEI. A) CNF sample. B) CTF sample. C) SU8NF sample. D) SU8TF sample.
Figure 4-15. Fluorescence Microscopy pictures of neurons on CNF, CTF, SU8NF, SU8TF samples treated with 0.001% PEI. A) CNF sample. B) CTF sample. C) SU8NF sample. D) SU8TF sample.
Figure 4-16. Fluorescence Microscopy images of neurons on CNF, CTF, SU8NF, SU8TF samples treated with 0.0001% PEI. A) CNF. B) CTF. C) SU8NF. D) SU8TF.
Figure 4-17. Bar chart representing the percentage of live cells over the total number of cells on the four types of samples that underwent four different types of treatments (plasma, 0.1% PEI, 0.001% PEI, 0.00001%PEI). For each sample the percentage has been separately calculated for both the stripes and the background co-present in each picture. The stars on top of the white lines indicate which values are significant in relation to the various types of stripes and the respective background within each different group treatment (p<0.05).
Figure 4-18. Bar chart representing the percentage of live cells over the total number of cells on the four types of samples that underwent four different types of treatments (plasma, 0.1% PEI, 0.001% PEI, 0.00001%PEI). For each sample the percentage has been separately calculated for both the stripes and the background co-present in each picture. The stars on top of the white lines indicate which values are significant in relation to the same type of stripe treated in different way (plasma, 0.1%PEI, 0.001%PEI, 0.00001%PEI) and same type of background treated in different way (plasma, 0.1%PEI, 0.001%PEI, 0.00001%PEI) (p<0.05).
Figure 4-19. Bar chart showing the results related to neuron's cell body average size on CNF and CTF obtained from NeuriteQuant. The stars indicate which values are significantly different according to t-test analysis.
Figure 4-20. Bar chart representing the data obtained from NeuriteQuant analysis in relation to the neurite length per cell, the number of neurites per cell and the average length of each neurite of neurons cultured on CNF and CTF for 3 DIV. The stars indicate which values are significantly different according to t-test analysis.
Figure 4-21. Bar chart showing the number of branches per cell and the number of branches per neurite of neurons plated on CTF and CNF treated with plasma, 0.1% PEI, 0.001% PEI and 0.00001% PEI, respectively. The stars indicate which values are significantly different according to t-test analysis.
Figure 4-22. Bar charts representing the percentage of live cells and the cell average size obtained from the NeuriteQuant analysis performed on neurons cultured for 3 DIV on CNT and CNF samples. The stars indicate which values are significantly different according to Tukey analysis.
Figure 4-23. Bar chart representing the values obtained for the neurite length per cell, the number of neurites per cell and the average length of individual neurite of neurons grown for 3 DIV on CNT and CNF samples. The stars indicate which values are significantly different according to Tukey analysis.
Figure 4-24. Bar chart showing the number of branches per neurite and per cell of neurons cultured on CNF and CNT samples for 3 DIV obtained from NeuriteQuant analysis. The stars indicate which values are significantly different according to Tukey analysis.
Figure 4-25. Pictures after NeuriteQuant analysis of neurons at 3 DIV on CNT(3LBL), CNT(9LBL), CNF plasma treated and CNF treated with 0.00001% PEI. A) CNT(3LBL). B) CNT(9LBL). C) CNF_plasma. D) CNF_0.00001% PEI.
CHAPTER 5
CONCLUSION AND RECOMMENDATIONS FOR FURTHER WORK

In this study we analyzed how neuronal morphology changes when surface modifications are applied to traditional substrate for cell culture. At first we investigated neurons on layer by layer (LBL) SWCNT/PEI modified surfaces, specifically fabricating two types of samples where one was made by only 3 layers of CNT/PEI and the other by 9 layers. The images obtained through fluorescent microscopy were analyzed using NeuriteQuant software and the results showed that almost no differences in neurite outgrowth and branching could be detected among the two types of CNT surfaces and, moreover, the neurons cultured on the control (only-PEI-treated surface) performed much better. These results contrast with our initial hypothesis that, increasing the number of layers, the neurite outgrowth would have improved. Such expected results were closely linked to the expectation of a much more significant increase in roughness, when moving from the 3 layers to the 9 layers, which is not what we detected. In fact the difference in roughness between the 3 layers and 9 layers was only of few nanometers that was not big enough to affect cell development. On the other end we can state that, the higher performance detected on neurons grown on PEI compared to PEI/CNT surfaces, can be related to a decrease of positive charges on PEI/CNT surfaces due to the negative charge of the CNT buffering the positive charge of PEI. Another important finding is related to an increase in branching on neurons cultured on CNT over long time. In fact, if the measured neurite length on CNT and PEI was found to be almost the same either at 3 or 7 DIV, the number of branches increased. What we hypothesize is that the nanostructured surface was not rough enough to affect proximal neurites
(bigger in diameter) but was able to provide topographical cues to shorter and smaller branches (distal neurites) that can originate from them.

In the second part of this study, carbon nanofibers (CNF), SU-8 nanofibers (SU8NF), SU-8 thin film (SU8TF) and carbon thin film (CTF) were used as surface modifications. These samples were treated with different concentration of PEI because preliminary results had shown very high cell mortality at standard PEI concentration (0.1%). The results showed that neurons grew exceptionally fine on these materials when they were treated with the lowest PEI concentration (0.00001%) or only plasma treated. The hypothesis that we proposed was that PEI, at the highest concentration, would accumulate on the tortuous structure of the nanofiber mat so that it would become toxic for cells. This hypothesis can properly justify why almost no cells survived on the nanofibers but a possible toxic interaction between PEI and SU-8 must be introduced in order to justify the fact that very low cell viability was also detected on SU-8 thin film, an almost totally flat surface. Moreover, NeuriteQuant analysis of CNF and CTF samples showed that neurons had more and longer neurites on the flat carbonized film than on the carbonized nanofibers. This result was totally unexpected and is very difficult to understand with the current data available. For that reasons more investigations on surface chemistry of the CTF, treated or not with PEI, as well as of the SU-8 material interacting with PEI, would be of relevant importance for a correct interpretation of these results.

Finally we compared the results obtained for the CNT and CNF surfaces at 3 DIV and we found that, in terms of neurite outgrowth and branching, neurons did better on the rougher CNF compared to the less rough CNT surfaces as expected. Our next step
would be to investigate how neurons would behave at 7 DIV on CNF, SU8NF, SU8TF and CTF. Moreover cell adhesion tests would be very useful in order to understand at which extent the neurons are able to adhere and stick to the surface of these samples.

In conclusion our results show that neurons can properly grow and develop on SU-8 photoresist as well as on carbon materials with a better preference for thin carbonized film, followed by carbonized nanofibers and carbon nanotubes. For these reasons we can hypothesize that SU-8 is a biocompatible material with possible future applications in tissue engineering. In regard to the carbon materials here investigated, the good results obtained might guarantee the success of their utilization in several different applications where good neuron-surface interactions are needed. For example there are very good chances that CNT and CNF could be used as electrode-surface modification for MEAs in order to reduce the impedance and allow better signal recording while enhancing cell growth and development.
LIST OF REFERENCES


H. Gao, Y. Kong, and D. Cui, “Spontaneous Insertion of DNA Oligonucleotides into Carbon Nanotubes,” no. Figure 2, pp. 2–4, 2003.


110


BIOGRAPHICAL SKETCH

Valentina majored in biomedical engineering. She took part in the ATLANTIS double degree program in collaboration with Politecnico di Milano and University of Florida, receiving her master’s degree in the summer 2013.