Investigation of Neuronal Affinity to Photoresist Derived Carbon: Study of Differentiation and mRNA Expression in PC- 12 cells

A Thesis

Presented to the Worcester Polytechnic Institute
Department of Chemical Engineering

In partial fulfillment of the requirements for

Degree of Master of Science

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Thesis Title: Investigation of Neuronal Affinity to Photoresist Derived Carbon: Study of Differentiations and mRNA Expression in PC-12 cells

Degree: Master of Science

Date: May 04, 2007

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Abstract

Regenerative medicine holds promises for many neurodegenerative diseases such as Traumatic Brain Injury (TBI), a disorder that occurs when a sudden trauma causes damage to the brain, leading to apoptosis or necrosis of brain neurons. More than 5 million Americans suffer from TBI as a result of inability to regenerate damaged neurons. The aim of this project was to develop a biocompatible and electrically conductive substrate to promote growth and regeneration of neurons and for our long-term goal as a probe to record intracellular and multisite signals from brain. The substrate was fabricated by pyrolyzing a polymeric precursor –SPR 220.7 at temperatures higher than 700 ºC. Human Neuroblastoma cells - SK-N-MC, SY5Y and mouse teratocarcinoma cells P-19 were found to attach and proliferate on photoresist derived carbon film. Growth and differentiation of rat pheochromocytoma cell-PC12 that serves as a model for primary neurons was demonstrated. Initial examination of cell growth and differentiation was done by observing cell shape and size, and measuring the length of neurites after the cells were differentiated by NGF. Further characterization of cells cultured on photoresist derived carbon substrate was achieved by testing mRNA genes- GADPH and Tau. Findings from this investigative work would possibly help to study new approaches to promote neuronal growth and differentiation in damaged brain regions of people with TBI or in patients with other neurodegenerative disorders, such as Alzheimer's disease in regaining memories.

Acknowledgements

I would like to thank Dr. Hong S. Zhou for her encouragement over the past two years. She has taught me that the combination of hard work, dedication and high standards truly contribute to personal success. I wish to thank my co-advisor Dr. Jianhua Zhou who has been there for me every step of the way, with his abounding knowledge and constant willingness to help.

I would like to extend my thanks to Tie Zou and Kritsanapol Unge for training me on the cell culture, RT-PCR and all the techniques used in this research. Additional thanks to Jack Ferraro for helping me to set up the furnace that allowed me to fabricate the substrate.

Thanks to Prof. Camesano for letting me use Atomic Force Microscope and Prof. Thalladi for helping me in characterizing the carbon substrate.

And finally, I would like to thank my Papa- Mr. Raj Gupta, my Mom- Mrs. Kamlesh Gupta and my sister- Reetu, for their constant love and support

1. Introduction

Thousands of lives are influenced every year by loss of neuronal function as a consequence of neurodegenerative diseases or injury to the neuronal pathway of the central or peripheral nervous system. Neurons have ability to repair and regenerate in case of minimal damages. But, if major damage occurs to the peripheral and central nervous systems, the neurons fail to restore themselves leading to a loss of memory, impaired sensory responses, muscle disfunctioning.

Current efforts at solving this problem involve: (1) nerve grafting, which includes transplanting a normal donor nerve from a healthier organ; (2) surgical suturing involving end-to-end sewing of nerve stumps which results in increased tension across the repaired site. These procedures are however limited by the availability of donor tissue, donor site morbidity, multiple surgeries and partial recovery [1].

Tissue engineering offers various engineered biodegradable and non-biodegradable templates for regeneration of severed neurons. These engineered constructs are a promising alternative due to their mechanical strength, biocompatibility and chemical inertness. A variety of natural materials such as laminin, fibronectin and collagen are employed to improve the efficacy of these substrates. These adsorbed proteins promote the adhesion of cells by interacting with adhesion receptors present on cells. Several investigators are exploring a more direct approach, not depending upon a secondary mediator; they are focusing on covalent or physiochemical incorporation of adhesion-promoting biopolymers. These peptides are based on the primary structure of the receptor binding domains of proteins- fibronectin, laminin and often the corresponding linear and non-linear sequences can display similar receptor specificity and binding affinity as well

as signaling of cellular responses compared to the whole protein. Possible advantage of working with short adhesion peptides is that the peptides could be displayed in a manner such that nearly all of them would be available and active for binding [2] Massia SP et al. have shown that when a three amino acid sequence arginine-glycine-aspartic acid (RGD) from fibronectin, was immobilized by its amino-terminal primary amine via a glycyl spacer , approximately 106 copies per cell were required to stimulate cell adhesion, spreading and higher functions , whereas many more copies of the complete protein were required [3,4] . Both these natural and synthetic materials can support axonal migration and toxic effects however these materials induce undesirable immune response and lack mechanical stability.

The need of mechanical stable neural implant materials led researchers to explore the possibility of employing various metals and non-metals for nerve regeneration purposes. Till now, silicon is being widely explored material for the continuous monitoring, diagnosis and regeneration of neurons. Silicone tubes have been used for nerve restoration applications. They are non-biodegradable and impermeable to large molecules and created an isolated environment for nerve regeneration.[5] Researchers have successfully shown the organization of neural networks on silicone tubes functionlized with laminin, collagen and fibronectin gels. Fromherz et al have been working on electrical interfacing between the individual nerve cells and have been working to control the mechanical and electrical quality of cell-semiconductor contact and signal processing between neurons. A layer of silicon dioxide is grown on silicon to suppress to suppress electrochemical processes which results in silicon corrosion. These inert chips are then coated with biopolymers to affect cell adhesion [6]. However, the non-biodegradable

silicon imparts inflexibility and induces foreign body reaction resulting from excessive scar tissue.

Lack of insufficient bonding of silicon implant to desirable surrounding tissue and lack of inherent inflexibility, mechanical instability and lower electrical conductivity has led investigations of novel biomaterials that can overcome the limitations of synthetics implant device. There has been growing interest to use carbon for biomedical applications. Carbon especially in its fiber form has been successfully used in the orthopedic and neural implants due to its composition, high aspect ratio, electrical and physical properties [7]. Previous studies have provided evidence that carbon fibers possess ability to be functionalized with biomolecules has made them successful candidates to form scaffolds to from neuron hybrids. X. Zhang et.al have demonstrated the capability of functionalized vertical nanotube arrays as support platform for guiding neurite growth and forming synaptic ally communicative networks [8] . Pyrolzed carbon is also being investigated for its applications in artificial heart valves due to its ability to maintain a coherent interface with living tissue and its ability of being non-thrombogenic in blood [9-10].

In the present work, we show the successful growth and regeneration of various human and rat nerve cells on photoresist derived carbon. Carbon derived from photoresist can be easily patterned by lithography techniques to study the migration of axon along the carbon structure. Eliminating some neurons from the organizations and observing the regeneration of neurons to complete the network will enable in understanding the nerve restoration process at the injured site. We demonstrated the growth, proliferation and

differentiation phenomena on photoresist derived carbon films that were not functionalized with naturally occurring protein or synthetically modified biopolymers.

This may considerably reduce the undesirable immune response and the need of immunesuppressors that hinder with the normal functioning of implant devices. Other advantages of these carbon devices, derived from polymeric precursor include excellent biocompatibility, wide electrochemical stability, and availability in high purity, reproducibility, chemical inertness, and good thermal conductivity, dimensional and mechanical permanence [11-12]. In the present work, potentiality of carbon as a resultant of pyrolysis of polymeric precursor- SPR 220.7 for in vivo applications such as neural probes, neuronal guide paths for spinal cord injuries and castings for implanted devices is being investigated. Several human and rat; neuroblastoma (SK-N-MC, SY5Y) and carcinoma (P-19) cells were tested for their growth and proliferation on the carbon template. PC-12 (rat pheochromocytoma) cells can differentiate to neurons when subjected to differentiation-inducing substances. PC-12 cells have been widely used as a system for differentiation studies [13] . In this study, we examined the changes in cellular morphologies of PC-12 cells seeded on carbon surface and coated glass and differentiated with nerve growth factor. We also compared the GADPH, Tau and mRNA gene expressions in NGF induced PC-12 suspended on carbon and coated glass substrate by employing Reverse Transcription and Polymerase Chain Reaction and Western blot techniques [14].

2. Background

2.1. Neurons

A human body consists of billions of nerve cells. Neurons are the basic components of brain, peripheral, central nervous system and are responsible for carrying signals from the brain to the rest of the body. All nerve cells are derived as a result of differentiation of neural stem cells.

2.1.1. Anatomy

A nerve cell (neuron) consists of a cell body, axons and dendrites enclosed within a phospholipid bilayer membrane. They are specialized for processing and transmission of signals. Soma, the cell body of a neuron ranges from 3-18 micrometers in size. It consists of nucleus and other cell organelles for protein synthesis. Dendrites are the projections emerging from soma that bring information from neighboring neurons to the cell body. An axon is a cable-like cellular extension that carries information away from the cell body [6].

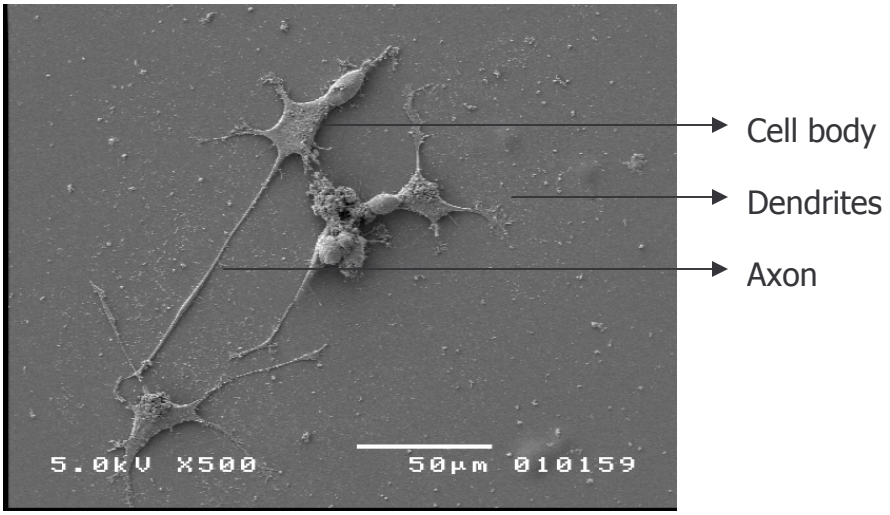


Figure 1: Illustration showing the anatomy of nerve cell

Glial cells are the other main constituent of nervous system with a ratio of ten glial cells to each neuron. They provide support and nutrition to the neurons and participate in signal transmission. Glia has important developmental roles, guiding migration of neurons in early development, and producing molecules that modify the growth of axons and dendrites. Schwann cells are involved in maintenance of axon and provide myelin sheath for insulation and protection of the axons [51]. The neurons and glial cells attach to the extracellular matrix (ECM) occurring naturally in the environment. ECM influences the morphology, proliferation and migration of the cells. Some of the ECM molecules include collagen, laminin and fibronectin. These molecules are present in the endonerium and basal membrane and contribute in formation of neural networks and axonal guidance in vivo and in vitro [25].

2.1.2. Neurite Outgrowth

Along with glial and Schwann cells , neurotropic factors like- nerve growth factors (GNF), brain-derived neurotropic factor (BDNF) and neurotrophins are present [26]. These neurotropic factors are responsible in inducing axon outgrowth and branching of neuritis. During the early stages of development, a high level of NGF is localized in the cells. After the initial stages, the level of NGF decline to an amount, suffiient for maintenance of cells. Receptors of NGF concentrate in the tip of the neurite and trigger signal transduction in the cells. The signaling pathways inhibit cell death and induce neurite extension, aiding in the growth of developing of injured cells. The signals generated by nerve cells are transmitted by axon to the contacting dendrites or cell body of another neuron. The signal is in the form of chemicals (neurotransmitter) that are transduced into electrical signals, which are regenerated actively along the cells. The signaling pathways induce actin molecules to polymerize on the leading edge of the growth cone, forming focal points, creating a driving force for growth cone extension [39].

Summarizing, the growth cones at the ends of neurite extensions are influenced by the extracellular matrix and growth factors. Cell adhesion molecules; contribute to the attachment, elongation and migration of cells on matrix in response to differentiation by NGF. This multicomponent system functions in normal development and during the regeneration of injured/damaged nerves [40].

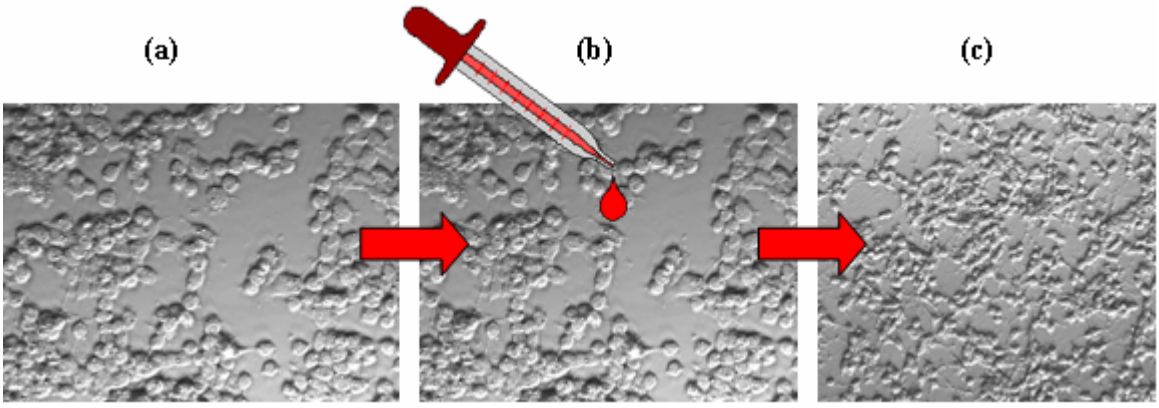


Figure 2: Cell Differentiation

(a) PC-12 cells from obtained from cell lines maintained in medium at 37ºC, 5%CO_2

(b) PC-12 cells injected with nerve growth factor at a concentration of 5µL/ml

(c) PC-12 cells demonstrating neurite outgrowth

2.2. Brain Injuries

Peripheral nerve lesions are common and serious injuries affecting 2.8% of trauma patients annually, and generally lead to lifelong disability Traumatic brain injury (TBI) disrupts the normal functioning of brain. It causes a wide range of functional changes affecting thinking, sensation, language, and/or emotions. It can also cause epilepsy and increase the risk for conditions such as Alzheimer's disease, Parkinson's disease, and other brain disorders that become more prevalent with age [1].

2.2.1. Epidemiology

In the United States, 360,000 people suffer from upper extremity paralytic syndromes on an annual basis, whereas in Europe more than 300,000 cases of peripheral nerve injuries occur annually [15].

Each year in the United States:

- approximately 1 million head-injured people are treated in hospital emergency rooms,
- approximately 270,000 people experience a moderate or severe TBI,
- approximately 60,000 new cases of epilepsy occur as a result of head trauma,
- approximately 50,000 people die from head injury,
- approximately 230,000 people are hospitalized for TBI and survive,
- and approximately 80,000 of these survivors live with significant disabilities as a result of the injury

2.2.2. Symptoms

Some symptoms are evident immediately, while others do not surface until several days or weeks after the injury.

Headache, mental confusion, fatigue, behavioral/mood changes, trouble with - memory, concentration, thinking are some of the symptoms associated with mild case of TBI. Moderate or severe TBI includes change in personality, nausea, dilation, slurred speech.

Other medical complications that may accompany a TBI include pulmonary (lung) dysfunction; cardiovascular (heart) dysfunction from blunt chest trauma; gastrointestinal dysfunction; fluid and hormonal imbalances; and other isolated complications, such as fractures, nerve injuries, deep vein thrombosis, excessive blood clotting, and infections [28].

Disabilities resulting from a TBI depend upon the severity of the injury, the location of the injury, and the age and general health of the patient. Some common disabilities include problems with cognition (awareness, thinking, memory, judgment, insight, and reasoning), sensory processing, communication (language expression and understanding), social function and behavior or mental health.

Many patients with mild to moderate head injuries who experience cognitive deficits become easily confused or distracted and have problems with concentration and attention. They also have problems with higher level, so-called executive functions, such as planning, organizing, and abstract reasoning, problem solving, and making judgments, which may make it difficult to resume pre-injury work-related activities.

Language and communication problems are common disabilities in TBI patients. Some may experience aphasia, defined as difficulty with understanding and producing spoken and written language; others may have difficulty with the more subtle aspects of communication, such as body language and emotional, non-verbal signals [23].

Other Long Associated Problems

Other long-term problems that can develop after a TBI include Parkinson's disease and other motor problems, Alzheimer's disease, dementia pugilistica, and post-traumatic dementia.

Alzheimer's disease (AD) is a progressive, neurodegenerative disease characterized by dementia, memory loss, and deteriorating cognitive abilities. Research suggests an association between head injury in early adulthood and the development of AD later in life; the more severe the head injury, the greater the risk of developing AD.Some evidence indicates that a head injury may interact with other factors to trigger the disease and may hasten the onset of the disease in individuals already at risk.

Parkinson's disease and other motor problems as a result of TBI are rare but can occur. Parkinson's disease may develop years after TBI as a result of damage to the basal ganglia. Symptoms of Parkinson's disease include tremor or trembling, rigidity or stiffness, slow movement, inability to move, shuffling walk, and stooped posture. Despite many scientific advances in recent years, Parkinson's disease remains a chronic and progressive disorder, meaning that it is incurable and will progress in severity until the end of life [1].

2.3. Nerve Regeneration

The incidence of spinal cord injury in the US is 11,000 per year and the prevalence is 250,000 to 400,000. The cost to support a patient with a spinal cord injury through his or her lifetime is estimated to cost $400,000 to $2.1 million depending on the severity of injury [1, 20].

Unlike a cut that heals, the central nervous system has limited ability to fix its damaged nerves, in contrast to the peripheral nervous system. When parts of the central nervous system are critically injured, the CNS cannot generate new neurons nor regenerate new axons of previously severed neurons. Severed CNS tips initially try to grow, but eventually abort and ultimately completely fail to regenerate. A look into this mechanism will reveal much about how and why the CNS works the way it does.

Remarkably, almost 90% of cells in the CNS are not even neurons. Rather they are glial cells, which play an important role in supporting neurons both physically and metabolically. They maintain the extracellular environment to best suit and nourish neighboring neurons. The CNS and PNS have two distinct types of glial cells, and they are what accounts for the discrepancy in regenerative ability.

Current surgical strategies developed for nerve repair involve- grafting and end-to-end suturing of the nerve stumps. Nerve stumps tend to retract and their primary suturing is possible only when no tension is generated. If the gap between the nerves is large, a template is used to bridge and guide the axons [23].

2.3.1. Autografts

Nerve autografts have been used for nerve regeneration. Even though only 50% of patients regain useful function through autografts, it still remains an effective technique for nerve repair. The success of autologous nerve repair is due to the presence of Schwann cells and basal lamina endoneurial tubes, which provide adhesion molecules to regenerating axons. The disadvantage of this procedure includes loss of function at the donor site, donor site morbidity and the need for multiple surgeries. In addition, some biological constraints cannot be overcome by the progress of microsurgery.

To counter these short comes of auto grafts; the replacement of an injured nerve with other person's nerve (allograft) may be performed. These nerves tend to come from cadaver donors, with limited time for the harvesting and use of the nerve. Allograft techniques overcome the size difference and loss of function problems faced when using autologous nerve grafts, but is limited in their own right. As a consequence of inserting a foreign tissue into the body, the human system rejects the transplant. To stop the rejection and retains the allograft, the patient must be on lifetime immunosuppressive drugs or the grafts must be chemically decellularized prior to implantation. rejects immune the transplant [1].

2.3.2. Tissue Engineering

Approximately 8 million surgical procedures are performed annually in the United States to treat tissue and organ deficiencies, and the associated medical costs proceed $400 billion annually.

The goal of tissue engineering is to replace diseased or damaged tissue with biologic substitutes that can restore and maintain normal function. Advances in the areas of cell and organ transplantation and material sciences have aided in the continuing development of tissue engineering and regenerative medicine. The inadequacies of aurografts and allografts in nerve injuries greater that 10mm in rats and greater than 30 mm for humans emphasize the importance of developing new technique for severe nerve injuries. The strategies for tissue-engineered nerve bring together a multidisciplinary team of physicians, engineers, biologists for a common goal of the creation for a nerve guide. These guides provide an optimal environment for the restoration of nerves, while reducing the number of necessary surgical procedures. The development of such devices could result in success rates that surpass those of autografts [20].

A biomaterial nerve guide can be placed at the injury site, with the proximal and distal nerve stumps at opposite ends of the tube. By creating such a bridge, the invasion of glial scar tissue into the gap can be prevented and the cells have a support system for migration and elongation. The regeneration of neural cells by such tissue-engineered systems occurs in basic stages. After injury, there is a posttraumatic response followed by the initial migration of cells into the tube. As the cells migrate and extend processes, they begin to differentiate into neuronal and glial cells, and neurties begin to elongate from the neuronal cell bodies. The final stages of regeneration are the remyelination of the newly generated axons by the glial cells.

Four major components are necessary for potential nerve constructs in nerve replacement (1) a scaffold for axonal migration; (2) support cells; (3) growth factors ; and (4) an extracellular matrix. The most appropriate combination and interaction of these

individual components is currently unknown. To better understand the challenges in the interrelationship of these components, each one will be reviewed separately. The scaffold, support cells and the ECM can physically guide the neurite extensions within the guide, and insertion of the scaffold also prevents the ingrowth of fibrous tissues. Neurotropic signals for neurite extension can be released by the scaffold itself and by the distal stump of the injured nerve. The growth cones on the tips of the neurons migrating form the proximal end will detect the gradient of tropic signals in the guide and will distinguish their directionality based on the chemotactic attraction. The conduit concentrates the signaling molecules that would otherwise diffuse into the large nerve gap and lead to neuroma. The mass of axonal branching is not sufficient for nerve repair; as its does not provide a direct axonal extension to the distal nerve stump.

Novel nerve guides created with different combinations of the four major components should possess several common characteristics. The nerve guides should be biocompatible, noncytotoxic, noncarcinogenic, nonimmunogenic and nonmutagenic. Flexibility, permeability and ease of surgical application should also be assessed in the creation of a nerve guide. Additionally, the conduits should be biodegradable, resulting in the complete breakdown of the material after a designated time in the host. The rate of such degradation should be in accordance with the specific axonal growth rate, allowing adequate time for the use of the guide a support structure followed by the slow breakdown of the guide. The site of the injury, type of species and the are of the species can influence the rate at which the axons will grow, creating a need for conduits with different derogative properties [23].

2.3.2.1. Extracellular Matrix Molecules

Attempts have been made to use a variety of conduits to bridge nerve gaps. Both natural and artificial materials are available for structure-supporting axonal migration. Natural materials appear to improve biocompatibility, decrease toxic effects and enhance migration of support cells. A variety naturally occurring materials – laminin, fibronectin, collagen, poly-lysine have been used. Poly-D-lysine is a common substrate for nerve cell culturing and cellular migration. It is used for preliminary analysis of cellular attachment because of its efficacy and economy. Several human and rat cells were found to attach to areas of polylysine coated glass coverslips [2].

Neurons demonstrate growth on laminin, as laminin is the major component of Schwann cell basal lamina. Laminin tested materials have higher levels of cellular attachment but do not specifically instruct the cells during development.

Similar to the positive outcomes of laminin coated substrate, coating with collagen or fibronectin increases cellular attachment. Collagen gels and collagen-based guides provide a matrix for cells and are effective in nerve regeneration. A three-dimensional collagen gel supplies the necessary mechanical support and the network of large pores that help in neuronal adhesion and survival. The interior of the guide can be filled with porous collagen sponges, or with magnetically aligned collagen fibers to stimulate fster axonal growth and recovery function.

Similarly fibronectin coated substrates enhances the regeneration of axons. As compared to collagen and laminin, fibronectin may be an essential component of glial cell attachment and proliferation. Modifying nerve guides with laminin or collagen has a positive effect on nerve regeneration. The nerve cells can attach more readily and more

specifically to these molecules, while sustaining the ability to detach for migration. Novel conduits made or coated with these materials may prove to be useful for improving peripheral and central nervous system regeneration [2, 16- 17].

2.3.2.2. Synthetic Polymers

Biodegradable synthetic polymers are advantageous because of their flexibility, as variations in their chemical or engineering properties may change biocompatibility, degradation behavior, porosity and mechanical strength. Copolymerization has been used to obtain materials with tailored characteristics in terms of degradation behavior, mechanical performance, thermal properties and wettability.

Cell adhesion to natural materials is based upon indirect recognition, that is, by proteins from the body fluids adsorbing nonspecifically to the materials surface and some subset of these adsorbed proteins, including fibronectin, fibrogen and vitronectin, promoting the adhesion of cells by interacting with the corresponding adhesion receptors. As a more direct approach, one that permits a greater degree of control by not depending upon a secondary mediator, several investigators have explored the covalent or physiochemical incorporation of adhesion-promoting oligopeptides and oligosaccharides.

Extensive research has been performed on the incorporation of adhesion-promoting oligopeptides into biomaterial surfaces. This peptide is based on the primary structure of the receptor-binding domains of proteins such as fibronectin and laminin, and often the corresponding linear or cyclized sequences can display similar receptor specificity and binding affinity, as well as signaling of cellular responses, compared to the whole protein.

Early work demonstrated an important advantage of working with short adhesion peptides, rather than the complete protein, that is, the peptides could be displayed in a manner so that nearly of them are available for binding to the cell-receptors present on the surface of the cells. When a bioactive tripeptide from fibronectin, the sequence RGD (amino acid single letter code), was immobilized by its amino-terminal primary amine via a glycyl spacer, approximately 105 copies per cell were required to induce cell adhesion, spreading, focal contact information and cytoskeletal organization, whereas many more copies of the complete protein were required, presumably due to unfolding of the protein associated with adsorption or absorption of the protein in an orientation such that the receptor-binding domain was not sterically available.

Among other bioresorable materials, aliphatic polyesters and copolyesters have been reported as suitable for nerve regeneration. They include- poly-L-lactic acid, poly-glycolic acid, poly-L-lactide-co-glycolide and poly-caprolactone[3-4].

2.3.2.3. Non-Degradable Materials

2.3.2.3.1 Silicone

Silicone tubes have mostly been used for nerve regeneration. They are non-biodegradable and not permeable to large molecules, and create an isolated environment for nerve regeneration, which allows the study of the effect of different extra-cellular matrix (ECM) analogous for axonal elongation. Typical controls are autografts, empty silicone tubes or silicone nerve guides filled with saline. Chen et al. showed that silicone

nerve guides filled with collagen, laminin- and fibronectin-based gel resulted in a more mature organization of regenerating axons when compared to controls. Other reports have been focused on the control of neuronals outgrowth by filling silicone tubes with longitudinal oriented collagen or laminin gels. However, the non-biodegradable tube elicited an inflammatory response which caused the formation of a fibrotic capsule around the guide, leading to chronic nerve compression [5- 6].

2.3.2.3.2. Carbon

Silicon is still widely used for neural implants because of its capable application as electrodes needed for undisrupted monitoring, diagnosis, and treatment of neural tissue. But the lack of sufficient bonding of silicon implants to desirable surrounding tissue and the inability to obtain necessary inherent properties have led to the investigations of novel materials.

Carbon Nanofibers

The discovery of carbon nanotubes has been rapidly developing as a platform for a variety of uses including biomedical applications. Researches have proved the biocompatibility of carbon fibers with physiological cells and tissues. Nano-dimesioned fibers have excellent conductivity; high conductivity is a promising property as electrical stimulation has been shown to be beneficial for nerve functions and for regeneration. The size of carbon nanofibers contributes to their strength and high conductivity, and their

nano dimensions correspond to the size of physiological proteins. It has been showed that increase in conductivity correlates with its decrease in foreign body response. The outstanding electrical and mechanical properties of carbon nanofibers lend themselves to potential applications as central and peripheral neural biomaterials [9].

Carbon nanotubes (CNT) are cylindrical structures of diameter of the order of few nanometers and length can extent to few millimeters. Carbon nanotubes can be categorized as –single walled nanotubes SWNT and multiwalled nanotubes (MWNT) based on number of carbon atoms present around the tube and the offset where the carbon wraps upto. CNT are synthesized by catalytic and chemical vapor deposition. CNT are separated into two groups, those considered to be conventional (with diameters greater than 100nm, specifically 125 and 200 nm), and nanophase (with diameters of 100 nm or less, specifically 60 and 100 nm). In each group of fibers a high energy (125-140 mJ/m2) and low surface energy (25-50mJ/m2) fibers are present. The high surface energy fiber is obtained by pyrolytic stripping of the carbon fiber to remove the outer hydrocarbon layer [33].

Carbon nanotubes are fabricated by- arc-discharger, laser ablation and chemical vapor deposition (CVD). CVD is the widely used method to prepare CNTs. This process involves reacting a metal catalyst with a hydrocarbon feedstock at high temperatures (>700ºC) to produce CNTs which depending on the reaction conditions can create a wide variety of lengths and widths. Nanotubes produced using this method commonly have metal catalysts or carboneus deposits on the outside of the nanotube. Since metal catalysts such as nickel can be used for growing CNTs. An additional step of purification of carbon nanotubes is desired for biomedical applications. Refluxing carbon nanotubes

in an oxidizing acid – nitric acid, which is the most popular method. This procedure oxidizes and removes the metal catalyst and carbonaceous deposits form the tubes. However, washing with acids can attack the more reactive ends and creates carboxylic acid groups. This issue can be resolved by tuning the surface chemistry of the tubes [34].

Areas where carbon nano tubes can be used relevant to tissue engineering are cell tracking studying cellular behavior, augmenting cell behavior and creating tissue matrices.

Cell tracking involves tracking implanted cells and monitoring the progress of tissue formation .Labeling implanted cells would help evaluating the viability of the engineered tissue and understanding biodistribution and migration pathways of transplanted cells. CNT possess many properties desirable for optical detection. They possess optical transitions in the infra red that minimize the interferences. Infra red spectrum between 900 and 1300 nm is an important optical window for biomedical applications. CNT can be modified with radiotracers for gamma scintigraphy. CNT can provide image enhancement for a variety of modalities and may be used as NIR fluorescent labels [33].

The ability to monitor cellular physiology – ion transport, enzyme interactions, protein and metabolite secretion and cellular behavior – matrices that could aid in better desiging of tissues. Because of the unique electronic structures, carbon nanotube electrochemical sensors can potentially simplify the analysis of redox-active proteins and amino acids allowing cell monitoring in tissues. By assembling carbon nanotubes between two electrodes, a field effect transistor forms with the nanotubes.

Carbon nanotubes impact tissue engineering for structural support. Popular synthetic polymers- PLGA and PLA have been used for tissue engineering, they lack the necessary

mechanical strength. Additionally, these polymers cannot be easily functionalized in contact to carbon nanotubes, which can readily be functionalized. Carbon nanotubes have the potential for providing the needed structural reinforcement for tissue scaffolding. Carbon nanotubes are put into a host of synthetic polymers and various biolpolymers.

Several cell types have been successfully grown on carbon nanotubes. SWNT are blended with collagen to support smooth muscle cells. Price et al. have implicated carbon nanofibers as an important material in the future design of orthopedic/dental materials. They performed the cytocompatibility study of carbon nanofibers and polymer casts that promoted osteoblast adhesion and decreased fibroblast adhesion was observed on PLGA casts. Webster et al. also provided the evidence of greater adhesion of osteoblasts and neurons on CNs. Because MWNTs have diameter of 100nm, they can possibly be used to mimic neural fibers. If the nanotubes are placed in an array, they can be used to form neural networks.

Zhang et al used a more controlled approach for cell patterning consisting of microfabrication method to define regions for cell attachment. They created a micropatterned substrate using conventional optical lithographic principles. They investigated the effect of curvature on neuronal guidance. Curved lines are crucial in determining the direction of axon growth into target region. It has been established that in vitro process outgrowth associated with guided network formation from immobilized neuron soma and onto the substrate is curvature dependent

Gabay *et. al* showed well defined engineered cultured neuronal systems forming on high density carbon Nanotubes Island. Self organization enables the system to achieve optimal self assembly rather than forces unstable organization. Due to the super

electrochemical properties of CNT paasive electrical contacts to the CNT islands allows high quality neural recording [33- 35].

Photoresist Derived Carbon Film

Photoresist are included for fabricating microelectromechanical systems. Pyrolysis of the photoresist material in an oxygen-free atmosphere leads to the formation of carbon leaving behind volatile materials. Positive photoresists are based on novolak/diazonaphthoquinone resins consisting mainly of aromatic polymers that are extensively cross-linked and rendered by exposure to UV light. Negative resist consist of a mixture of aliphatic and photosensitive cross linking agent that cross links the polymer upon exposure to UV light.

Photoresist-derived carbon is a silicon wafer that has been layered with photoresist to a certain thickness and heated at very high temperatures to carbonize the photoresist. This type of carbon is extremely strong. The sp3 hybridized orbital of the carbon has been known to exhibit diamond-like characteristics. The only feature that outshines the strength of the carbon is the ability to make complex-shaped electrodes. Photoresist-derived carbon is susceptible to photolithographic patterning. These carbon electrodes provide reasonable electrical conductivity along with a strong resistance to corrosion from electrolytes. These features make photoresist-derived carbon very attractive to the field of Microelectromechanical Systems (MEMS)

By changing the process parameters like soft and hard baking time, temperature, environment , photoresist derived carbon permits a wide variety of interesting nre MEMS

application that employ structures having a wide variety of shapes , resistivity and mechanical properties. Researches have shown that photoresist derived carbon electrodes show excellent electrochemical kinetics comparable to other available carbon types for selected electrochemical reactions in aqueous and non-aqueous electrolytes.

Advantages of photoresist derived carbon includes excellent biocompatibility, a very wide electrochemical stability window, availability in high purity, reproducibility, very fast electrode kinetics for simple redox systems, chemical inertness, good thermal conductivity , dimensional and mechanical stability and low weight.

Pyrolyzd carbon has been extensively used in artificial heart valves and has found some applications in various implants due to its ability to maintain a coherent interface with living tissue and because pyrolyzed carbon surfaces in blood are non-thrombogenic. The advantage of using photoresists as the starting material for carbon electrodes include the fact that photoresists can be patterned by photolithography techniques resulting in different geometeries including microstructures and nanotexture [37-38].

2.3.2.4. Directionality

The platform that is supplied by tissue-engineered constructs directs the extension of regenerating axons. Biomolecules- laminin and collagen provide support and guidance; the structure of the conduit can be used for physical guidance. The neurite outgrowths contact the surface of the guide and migrate along in response to the physical and biochemical cues to form a neuron pathway. Patterened biomaterials are currently being developed to influence the directionality of regenerating nerves. These micropatterns

exist in the form of both physical grooves in the material surface and aligned molecular signals.

Techniques like compression molding and casting are used to produce grooved materials. By modifying the cast the pattern dimensions can be altered for optimal neurite outgrowth. Similar to the dimensions of the nerve guide channels, the dimensions of the groove should be large enough for at least one cell body, but still capable of providing physical guidance. With proper spacing the neurites grow along the groove, as opposed to branching in varying directions. The alignment of the neurites can be further influenced by the addition of Schwann cells and laminin.

Material surfaces can be micropatterned with proteins or sequences to promote the directionality of neurite growth. Using simple coating procedures or microlithograpgy adhesive molecules can be attached to the material. Proteins used in such procedures for nerve guides include laminin, fibronectin, and vitronectin. The cell bodies become oriented on the laminin and the integrins concentrated in the growth cones allow the interaction with the laminin molecules [39- 40].

2.4. Cell types

Various cells, including established cell lines, and primary cells have been employed in the evaluation of biomaterials for nerve regeneration. The rat neuron-like pheochromocytoma (PC-12) cell line has been used as an in vitro model. This adrenal gland tumor cell line is stable and homogenous with differentiating capabilities. In response to nerve growth factor (NGF), PC12 cells extend neurites. Although these cells resemble sympathetic neurons, they cannot be used as an exact model of developing neurons and are best used as in initial cell studies [13].

2.5. Molecular Study

2.5.1. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

It is desirable to quantify the performance of tissue-engineered constructs in terms of in vitro and in vivo characteristics. Cell adhesion is normally studied by using microscope, in order to complete the characterization, it is necessary to measure the performance of the constructs at the molecular level.

Classical Northern blot analysis for measuring mRNA requires too many cells. The PCR in combination with prior reverse transcription (RT-PCR) of the mRNA of interest provides a means for measuring gene expression using as few as one cell. When RT-PCR is performed, the reliability of the data can be highly subjective due to the efficiency of both RT and PCR steps. This method is quiet sensitive for comparing mRNA from single cell. RT-PCR can also be used to compare mRNAs in different samples, to characterize levels of mRNA expression and to distinguish between related mRNAs. PCR is generally a three-step process, with denaturation, annealing and elongation steps, with temperatures that vary and are subject to a number of considerations that should be determined empirically. The number of cycle depends on the amount of target present and the efficiency of the reaction.

In the first phase of RT-PCR, that is reverse transcription step (RT) complementary DNA (cDNA) is made from messenger RNA (mNA) template using deoxyribonucleotide triphosphate (dNTP) and DNA polymerase, reverse transcriptase (RT). DNA buffer is

introduced to the above mentioned components and the reaction is allowed to take place for an hour at 37ºC.

In the second phase of this procedure, following RT, cDNA is amplified by PCR. DNA polymerase is added to the forward and reverse DNA primers. The primer anneals to the RNA, and the cDNA is extended towards 5' end of the mRNA through the RNA-dependent DNA polymerase activity of reverse transcriptase. Primers can be either gene-specific or nonspecific. Random hexamer primers contain all possible nucleotide combinations of a 6-base oligonucleotide and bind to all RNAs present. Oligonucleotides consisting solely of deoxythymidine residues anneal to the polyadenylated 3' tail found most RNAs. The reaction mixture is heated to a temperature above 37 ºC, to facilitate sequence specific binding of DNA primers to the cDNA. Further heating is done to make double-stranded DNA from the primer bound cDNA. The reaction mixture is heated to higher temperature around 90 ºC to denature the DNA by separating the strands. The temperature of the reaction mixture is lowered to bind the primers gains and the cycle is repeated to make millions of copies [14, 44].

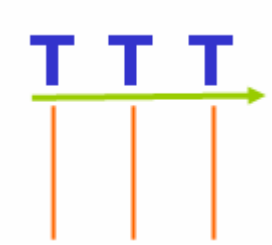


Figure 3: Commercially available Oligo dT Primer

Figure 4: mRNA strand extracted from cells

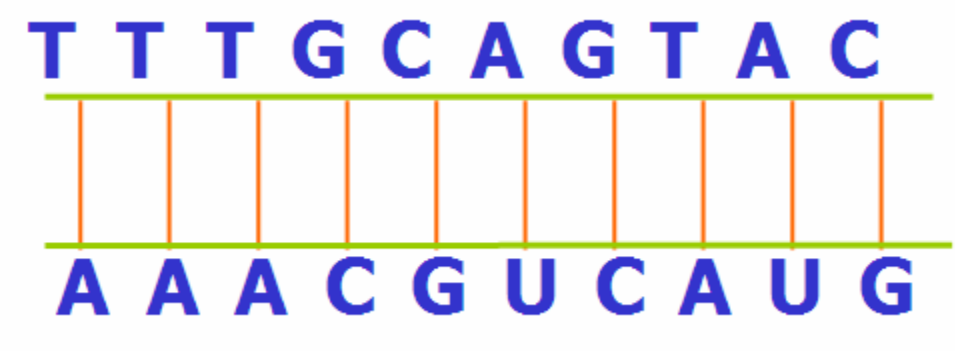


Figure 5: Reverse transcription reaction resulting in formation of new DNA from cDNA and mRNA

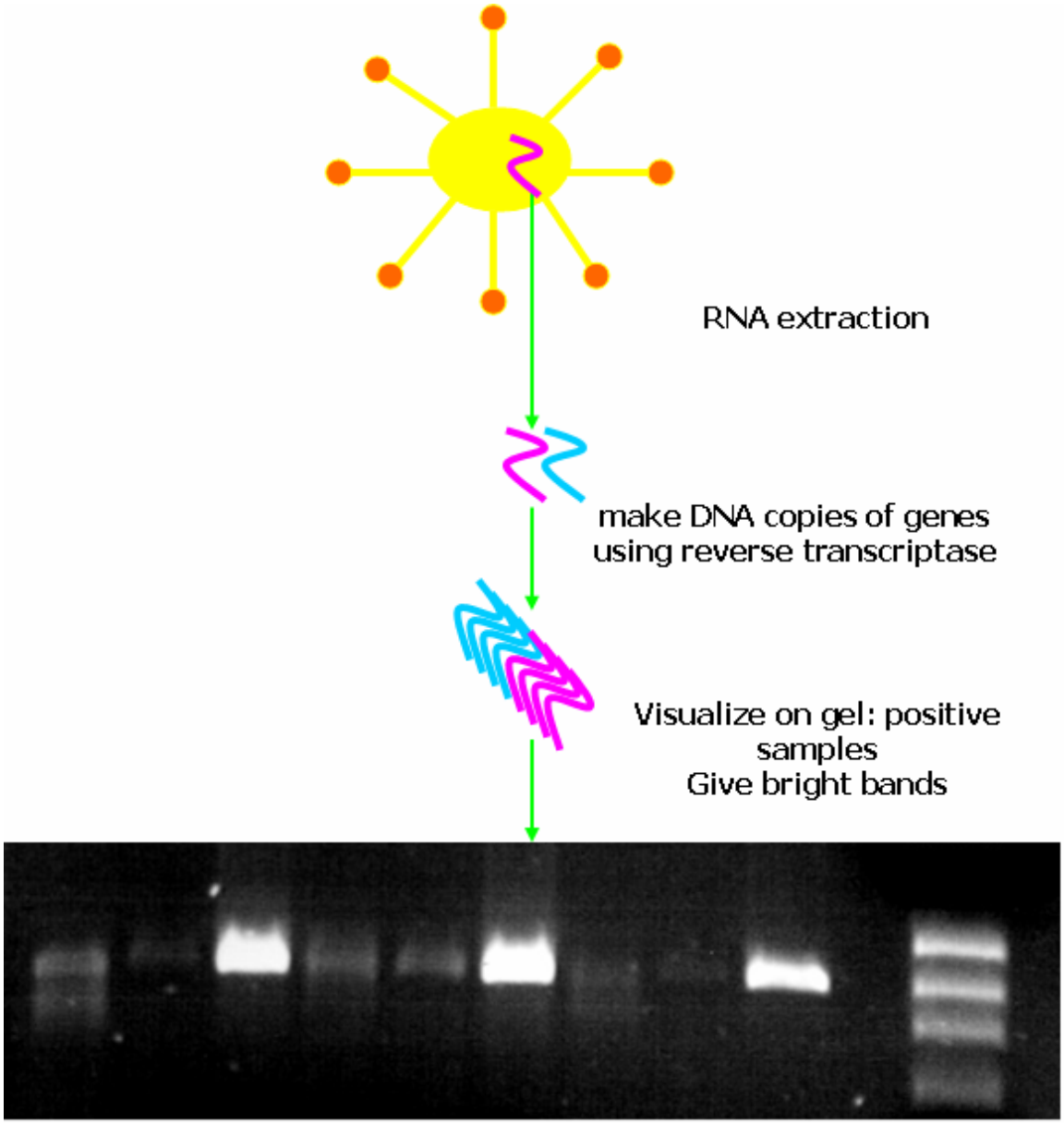


Figure 6: Illustration showing the steps involved in Reverse transcription polymerase chain reaction

2.5.2. Neuronal Gene Expression

Thirty to fifty percent of the approximately 10^5 mammalian genes specifically expressed in the nervous system; neuronal-specific genes must therefore account for the proper orientation and behavior of the 10^{12} neurons in the nervous system. The mechanism behind neuronal-specific transcription remains poorly understood, though significant ground has been gained in the understanding of the transcription of certain specific genes. There is probably not one sole mechanism by which genes achieve neuronal-specific expression; however, an examination of several well-studied neuronal-specific genes may reveal some insights into the phenomenon of tissue and cell type specific expression. Neuronal genes fall into two categories: those genes that have neuronal-specific promoters which are responsible for expression in the nervous system, and those genes containing generic promoters that achieve specifically via tissue-specific silencers [45].

2.5.2.1. Tau Gene

The human microtubule- associated protein (MAP) tau is a single copy gene and is abundant within axonal compartments of neurons. It is not found in either glial cells or in cell bodies, although it must be produced in the cell body and shipped to the axon, since the axon cannot support such production. Although tau does not directly crosslink microtubules, it promotes microtubule bundling through stabilization. Tau is important for neuronal cell morphogenesis, especially axonal maintenance, and is thought to make

short crossbridges between axonal microtubules, further stabilizing the structure. Its expression increases rapidly during development when axonal outgrowth occurs and decreases as the animal matures, thus, it is very important protein early in the development of the nervous system, and is not as essential once the networks are formed [47].

2.5.2.1.1. Tau-Gene Structure

The human tau gene contains sixteen exons, including one that is transcribed but not translated, designated-1. There are two CpG islands within the gene, which is typical of regulated genes; one associated with the promoter that overlaps exon-1, and another associated with exon 9. A 3'-untranslatd region of 4 kb, more than the size of the protein coding region. (1.4 kb) , exists at the end of the gene. Rat tau has two polyaenylation sites; one within the intron, 13/14 is retained in the mRNA at all stages of rat and human cortical develpmet. Tau mRNA is also responsive to retinoic acid; thus, the region around exon-1 might be important for regulation of the gene [48- 50]

2.5.2.2. Glyceraldehyde-3-Phosphate Dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GADPH) is an important enzyme in glycolysis and gluconeogenesis, whose cycles occur in the cytoplasm. GADPH is responsible for catalyzing the reversible conversion of glyceraldehyde 3-phosphate (GAP) and inorganic phosphate into 1,3-bisphosphoglycerate.

GADPH is a tetramer with a total molecular weight of 145 kD. Each subunit contains the coenzyme- NAD; each subunit contains 331 residues with molecular weights of 35.9 kD. In all four subunits there are both parallel and anti-parallel beta sheets; that are flanked by a layer of layer alpha helices. GADPH is composed of two folding domains. The first domain contains the residues 0-148, which are involved in NAD binding. This domain is composed of a beta-alpha-beta pattern with a central beta sheet covered on both the sides of the alpha helices. Whereas, the second domain contains the side chain residues 149-333, which are implicated in catalysis. The second domain is comosed of extensive anti-parallel beta sheets regions. This domain also contains an S-shaped loop of polypeptide residues 178-201 which are in contact with several amino acids in contact with NAD.

GADPH is an acidic protein. On the surface of the active site there is an excess of negative charge. However, at the entrance of the active site pocket residues Lys191, 211 and Arg 231give an excess positive charge. The side chains of the S-lop and co-enzyme-binding domain contain en evenly distributed negative and positive charge [52].

2.5.3. Gel Electrophoresis

Gel electrophoresis is a widely used method in the field of biochemistry and molecular biology to separate DNA/RNA fragments by virtue of their molecular weight. This is done by running the mRNAs through an electric field. Smaller molecules move faster and migrate at a farther distance than the larger molecules. Other than the length of the strands of nucleic acids, conformation of molecule is another important factor to be considered, to avoid this problem; molecules are usually separated using restriction

digest. Increase in agarose concentration reduces the migration speed and enables the separation of smaller molecules. Increase in voltage also results in faster migration, but is undesirable as it causes the melting of gel and denaturing the RNAs and DNAs.

Ethidium bromide is the most common dye used for gel electrophoresis. Under the UV light, it makes the molecules to glow [14].

3. Project Goal

The goal of nerve tissue engineering is to create an environment that allows for the regeneration of nerves. Photoresist derived carbon could provide the necessary support and guidance directly at the injured site. By experimentation with PC 12 cells and other human and mouse nerve cells, photoresist derived carbon may prove to be useful for nerve regeneration.

The objective of this work was to successfully fabricate carbon template by pyrolyzing a positive photoresist-SPR 220.7 of approximate thickness 10 μm. Different human and mouse cells were employed to investigate their affinity to the carbon substrate.

Main objective of this work was to demonstrate PC-12 cell differentiation.PC-12 cells were cultured on carbon substrate before treating them with nerve growth factor. Characterization was done by measuring the length of each neurite and number of neurites emerging from the cell soma. In order to complete the characterization and prove the normality of cells growing on the unfunctionalized carbon substrate, neuronal gene expressions were studied and compared to the genes expressed by cells grown on coated glass coverslips.

4. Procedure

4.1. Fabrication of Carbon Substrate

Carbon was derived from pyrolysis of photoresist coated on silicon wafer. A 2" diameter silicon wafer is cut into numerous chips of approximate length 15.6mm. Silicon chips were washed with 70% ethanol and then air dried. A clean silicon chip was placed in the spin coater. A layer of positive photoresist SPR 220-7.0 was applied to the silicon chip manually. The spin coater was run at 300 revolutions per minute (rpms) for 3 seconds to spread the photoresist on the wafer then run at 3000 rpm for 30 sec to fully situate the photoresist on the silicon chip. The process outlined in this source gives a thickness of eight to ten microns (μm). This thickness was sufficient to provide a dense and solid carbon surface.

There is always some degree of shrinkage; therefore a thickness of this size is required to have a considerable amount of solid carbon present after the completion of heating. The photoresist coating process was repeated four times to ensure that the desired thickness range had been reached.

Photoresists are classified into two types - a positive resist and a negative resist. Previous experiments suggest that both forms of photoresists under suitable conditions carbonize. The type of photoresist to be used in this process would depend on supply, of course, but it would be more efficient to start with the positive photoresist, if possible, as it would cut down on time needed to run the experiment and it would cut down on materials needed. However, if a negative photoresist were used, the sample created

would require treatment with UV light. Negative photoresist, being manufactured the way it is, will not ensure cross-linking of the carbon if it were not treated with UV light. If there was no cross-linking involved in the manufacturing of the carbon electrode, the carbon would lose much of its strength and would be more susceptible to deterioration among electrolytes. If a positive photoresist is used, this step would not be required as cross-linkage would already be certain.

Following the four coats, photoresist also required a soft bake step. Photoresist coated silicon chip was placed on a hot plate set at 95°C for five minutes and was left to cool naturally to room temperature.

The chip was placed in a Nitrogen gas atmosphere (at least 95% N2, as the nitrogen gas tanks are not completely nitrogen and may contain up to 5% Hydrogen). The gas atmosphere must be completely free of oxygen because oxygen would acquire dramatic photoresist shrinkage. The sample was then heated at a rate of 10°C per minute until the desired maximum temperature (600°C to 1100°C) had been reached. Once at the desired maximum temperature, the sample remained under heat at that temperature for one hour. The chip is allowed to cool in the nitrogen environment because at that temperature, oxygen would still induce dramatic shrinkage of the carbon. Once at room temperature, the chip with a layer of carbon was ready to reenter an oxygenated atmosphere.

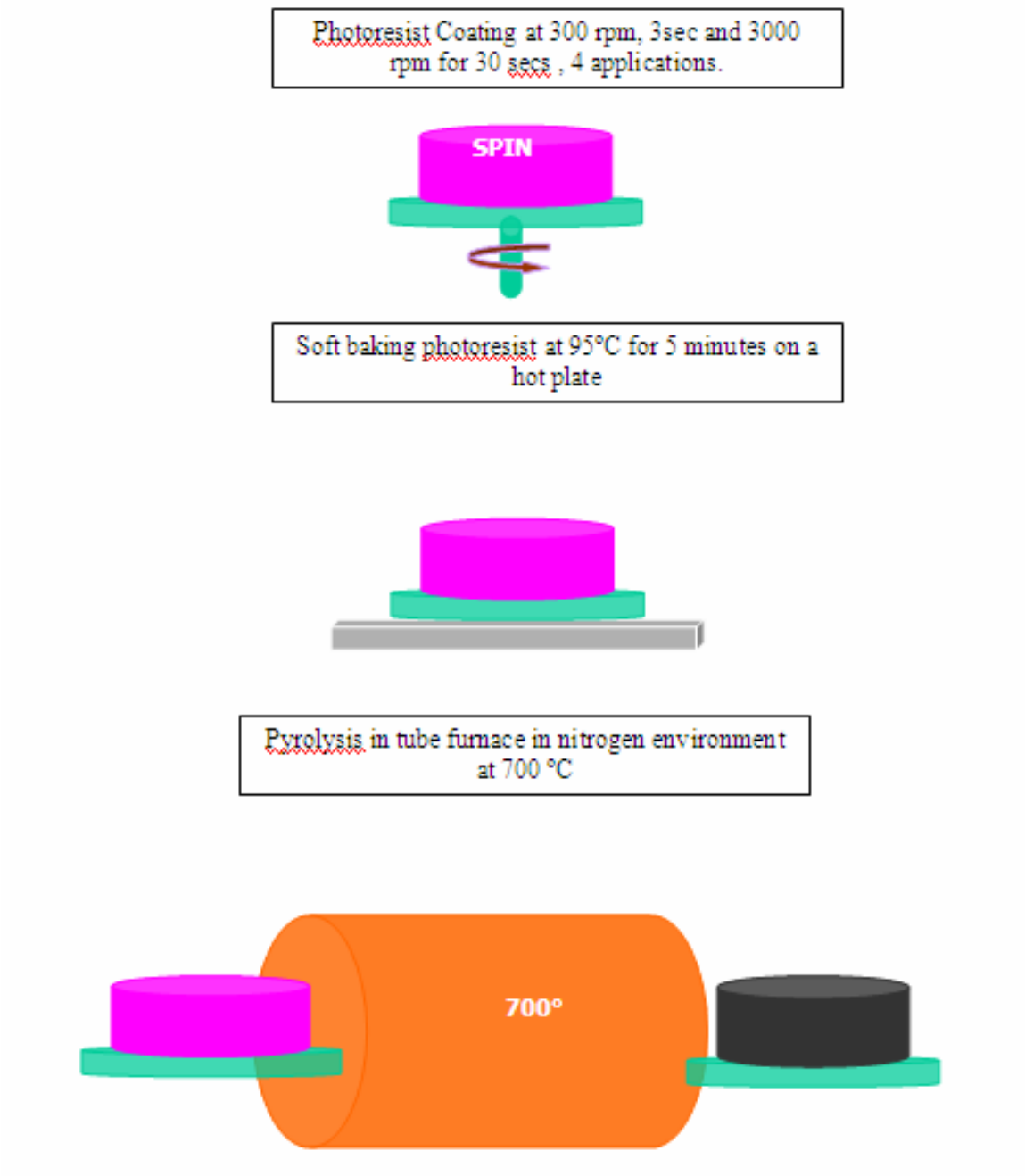


Figure 7: Illustration showing the steps involved in fabrication of Carbon

4.2. Substrate Preparation and Cell Culture

Carbon chips were washed with 70% v/v ethanol and sterilized under ultraviolet radiation for 45 minutes.

Human neuroblastoma cell lines (SK-N-MC, SY5Y), mouse teratocarcinoma cell line (P-19) , purchased from ATCC were incubated at 37°C with 5% CO2 in cell culture dishes. Cells were maintained in DMEM high glucose medium (Gibco 11495) supplemented with 10% fetal bovine serum (Gibco 26140-079) and 2% penicillin streptomycin (Gibco 15140-122). The cells were removed from the cell culture dish with 25% trypsin EDTA (Gibco-25200) and counted via a haemocytometer. Removed cells were centrifuged at 1000 rpm for 5 minutes. The pellet was suspended with fresh medium and transferred to sterilized carbon chips placed in 24 wells cell culture plate. pelleted and the appropriate concentrations were transferred to the sterilized carbon chip placed in 24 wells cell culture plate well.

4.2.1. Differentiation of PC12 cells

The rat pheochromocytoma cell line- PC12, obtained from ATCC were cultured in differentiation-inducing DMEM high glucose medium (Gibco-11495) supplemented with 0.5µL/ml Nerve Growth Factor (Gibco 13257-019). Nerve Growth Factor induces neurite outgrowth and formation of neural network between the cells.

4.3. Sample Preparation for Scanning Electron Microscopy

Differentiated and undifferentiated cells on photoresist derived carbon were fixed by immersion in 2.5% (v/v) glutaraldehyde in 0.5 M Na cacodylate-HCl buffer (pH 7.2) for 1 hr at room temperatute, the fixed samples were washed three times in the same buffer. Following the third wash the cells were post fixed for 1 hr in 1% osmium tetroxide (w/v) in the same buffer, washed three more times in the same buffer and left overnight at 4°C in fresh buffer. The next morning the samples were dehydrated through a graded series of ethanol to 100% and then Critical Point Dried in liquid CO2.

The carbon chips with the cells attached on the surface were affixed to aluminum SEM studs and sputter coated with Au/Pd (80/20). The specimens were then examined using an ETEC autoscan scanning electron microscope at 20kV accelerating voltage.

4.4. RNA Isolation

Differentiated and undifferentiated cells cultured on carbon were lysed in a culture dish by adding 1ml of TRIzol reagent (Invitrogen- 15596-026) to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. The homogenized sample was incubated for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of choloform per 1ml of TRIzol was added and the sample was transferred to sterile microfuge tube with secured caps. The tube was shaken vigorously by hand for 15 seconds and incubated at 15 to 30°C for 2 to 3 minutes. The sample was centrifuged to 12,000 X g for 15 minutes at 4°C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropyl alcohol. The sample was then incubated at 15 to 30°C for 10 minutes followed by centrifugation at 12,000 X g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol. The sample was vortexed and centrifuged at 7,500 X g for 5 minutes at 4°C. RNA pellet was briefly air dried for 5-10 minutes. RNA was then dissolved in RNase-free water and incubated for 10 minutes at 55°C in fresh buffer. The next morning the samples were dehydrated through a graded series of ethanol to 100% and then Critical Point Dried in liquid CO2.

4.5. RNA Quantification by UV Spectrophotometer

Hydrated RNA samples were vortexed and pulse spinned for 5 seconds. Dilution tubes were prepared by adding 198 µl of RNase free water to 1.5 ml tubes. 2µl of RNA was extracted from each sample and was mixed with RNase free water to make a total volume of 200µl, giving a 1:1001 dilution. All the dilutions were vortexed and pulse spinned for 5 seconds. A quartz cuvette was rinsed and cleaned with RNase-free water. Each RNA sample was pipetted into cuvette and absorbances were read at 320nm, (background), 280nm and 260 nm.

4.6. Reverse Transcription

1µl of oligo (dT) primer was added to the extracted RNA sample. The total volume was adjusted to 5µl by adding RNAase-free water to the tube. The tube was heated to 70°C for 5 minutes. The tube was then chilled on ice for 5 minutes before centrifuging briefly to collect the solution at the bottom of the tube.

4.7. Polymerase Chain Reaction

In a sterile, nuclease-free microcentrifuge tube, following mixture was prepared,

10X amplification buffer	4 µl
H2O	4.5 µl
MgCl2	4 µl
dNTP	1 µl
Reverse transcriptase	1 µl

5 µl of the RNA sample obtained after reverse transcription was added to the reaction mixture. The nucleic acids were amplified using the denaturation, annealing and polymerization times and temperatures listed below.

Denaturation	Annealing	Polymerization
1min at 25°C	1hr at 42°C	1min at 70°C

Table 1: Oligonucleotide primers for PCR Amplification

Gene	Primer Sequence
GADPH	F: 5'-CCG CTC ATT GCC GAT AG TG-3'
	R: 5'-GGC TGT GTG TCC CTG TAT-3'
Tau	F: 5'-CTG AAG CAC CAG CCG GGA GG-3'
	R: 5'-TGG TCT GTC TTG GCT TTG GC-3'

4.8. Gel Electrophoresis

Sample from the test reaction mixture was analyzed by electrophoresis through agarose gel. A solution of agarose was prepared by adding 1.5 grams of powdered agarose purchased from OmniPur (1594B97) to electrophoresis buffer (1 X TAE). The mixture was heated for 1 min in microwave. Ethidium bromide was added to the cooled agarose solution before pouring the solution into the mould. The gel was allowed to set compleletely for 15-20 mins. RNA samples were mixed with 0.20 volumes of the desired 6X gel-loading buffer before loading the sample mixture into the gel. A voltage of 120kV was applied and the gel was examined by UV light.

5. Results

5.1. Characterization of Carbon Substrate

The scanning electron microscopy (SEM) studies (Fig.1) showed that the photoresist derived carbon film had thickness of 10 μm, with no porosity at several high magnifications. Atomic force microscopy (AFM) reports the films having a relatively smooth surface with an estimated root mean square (rms) roughness of 2 nm (Fig.2). X-ray diffraction revealed the graphitic nature of the carbon film (Fig.3).

5.1.1. Scanning Electron Microscopy (SEM) images of Carbon

Scanning electron images were taken at various magnifications to have a clear idea of the surface structure.

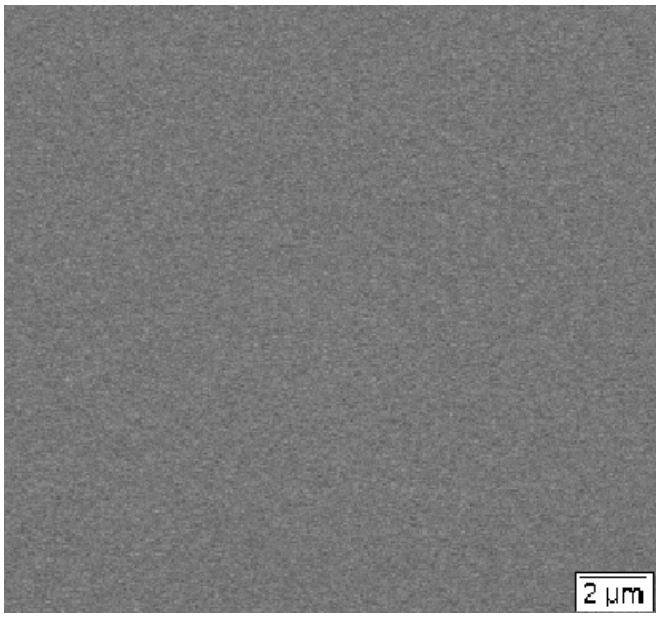


Figure 8: SEM Image of Carbon – 2 μm magnification

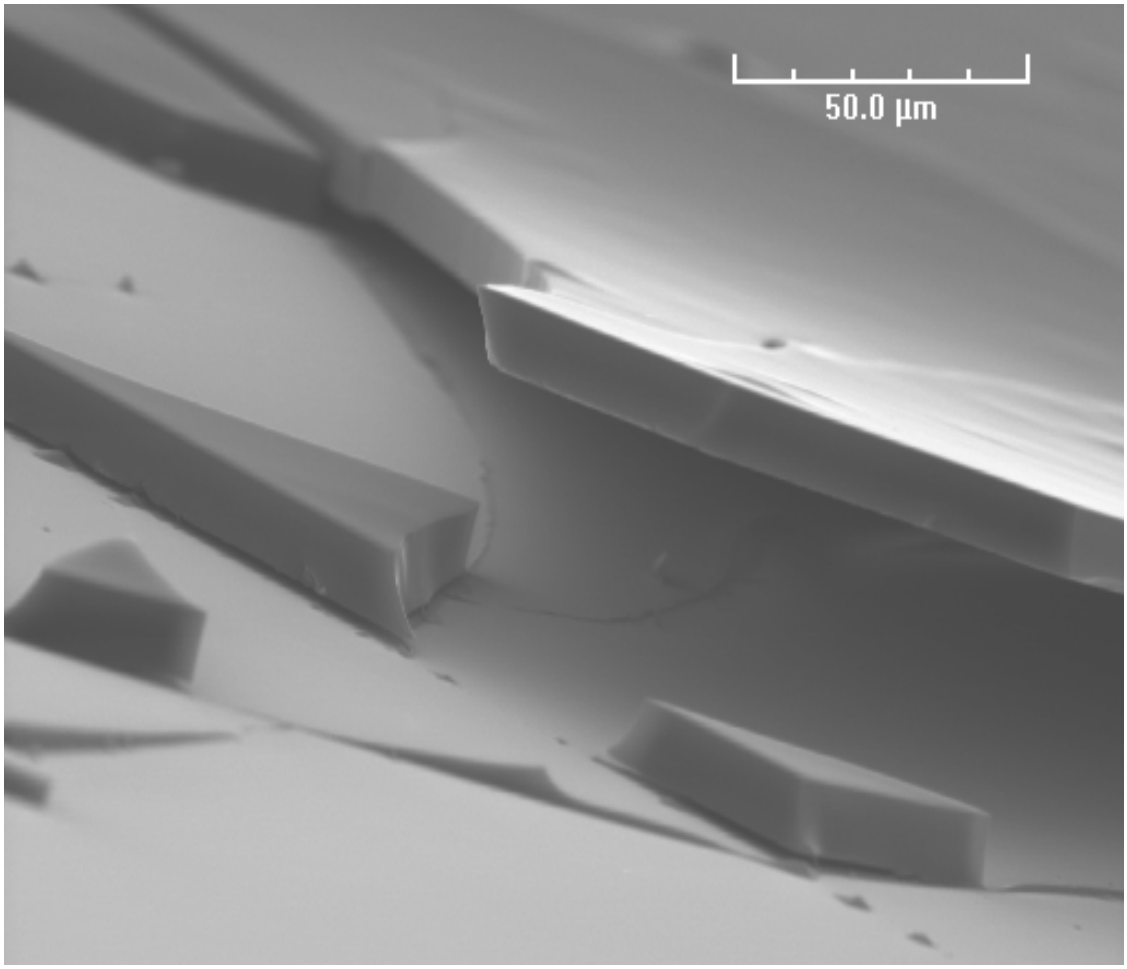


Figure 9: SEM Images of Carbon – 50 µm magnification

5.1.2. Atomic Force Microscopy (AFM) of Carbon

Atomic force microscopy was employed to calculate the roughness of the carbon substrate. Roughness was observed at various magnifications and the final roughness was calculated by rms value.

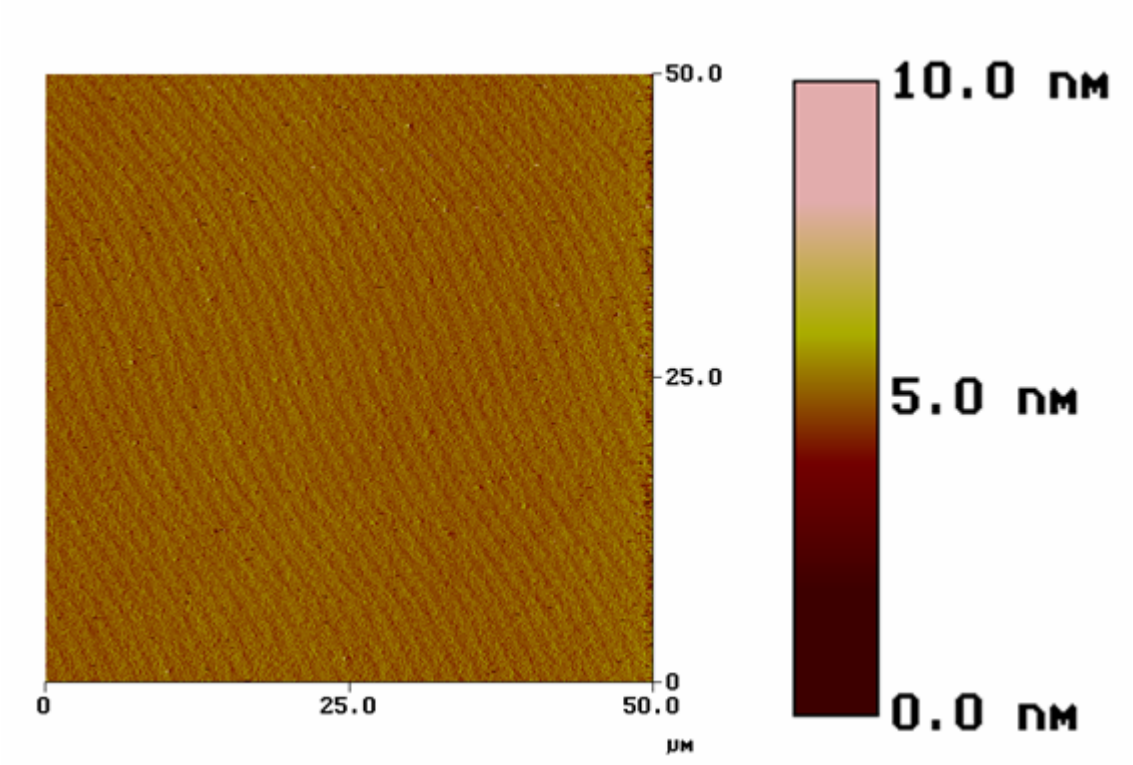


Figure 10: AFM Image of Carbon (50.00 X 50.00 µm)

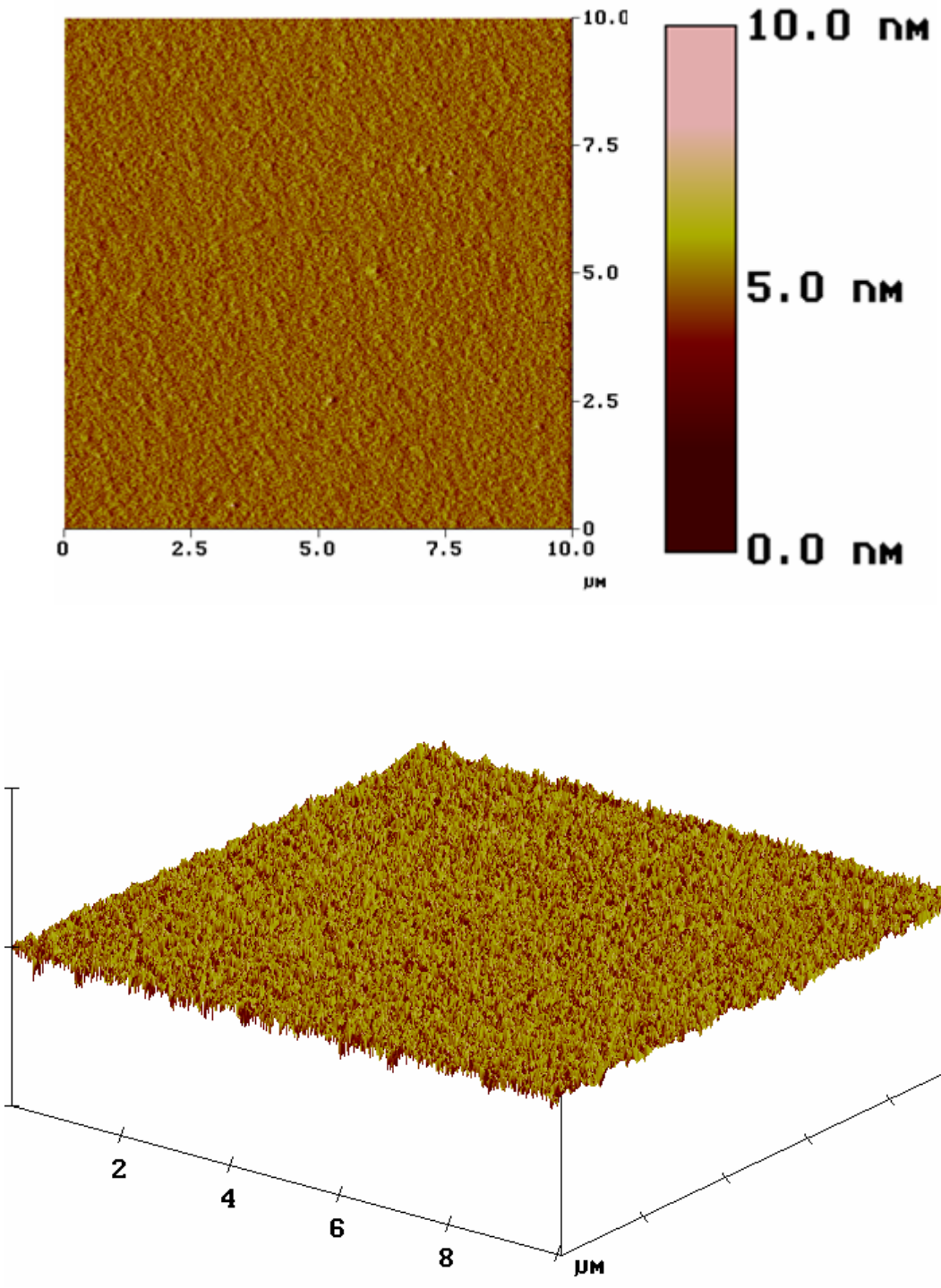
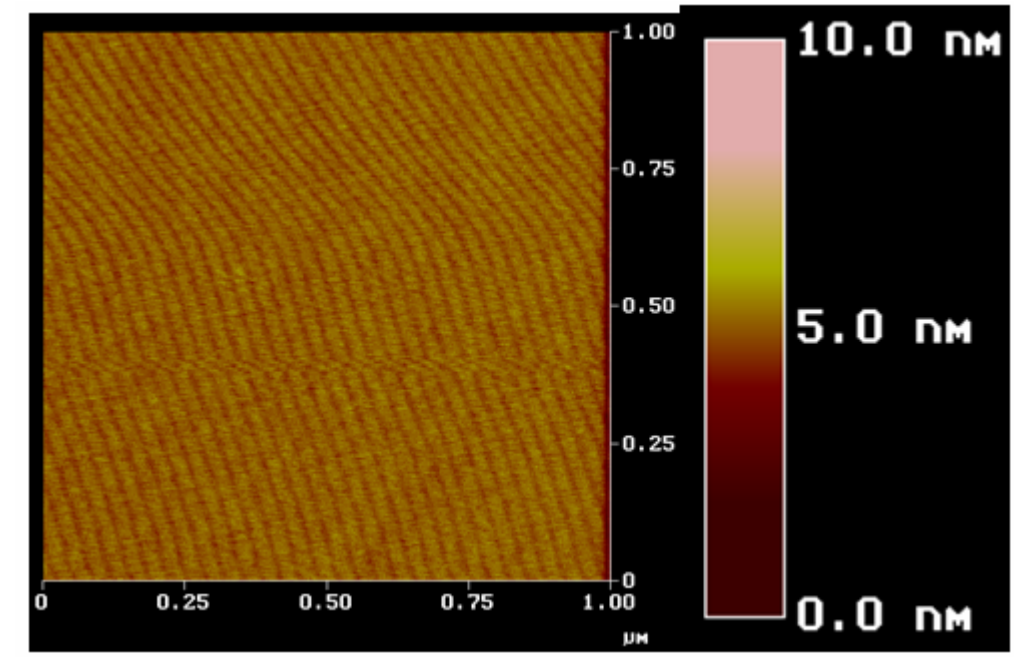


Figure 11: AFM Images of Carbon (10.00 X 10.00 [µm] Z-Max 10.0 nm)



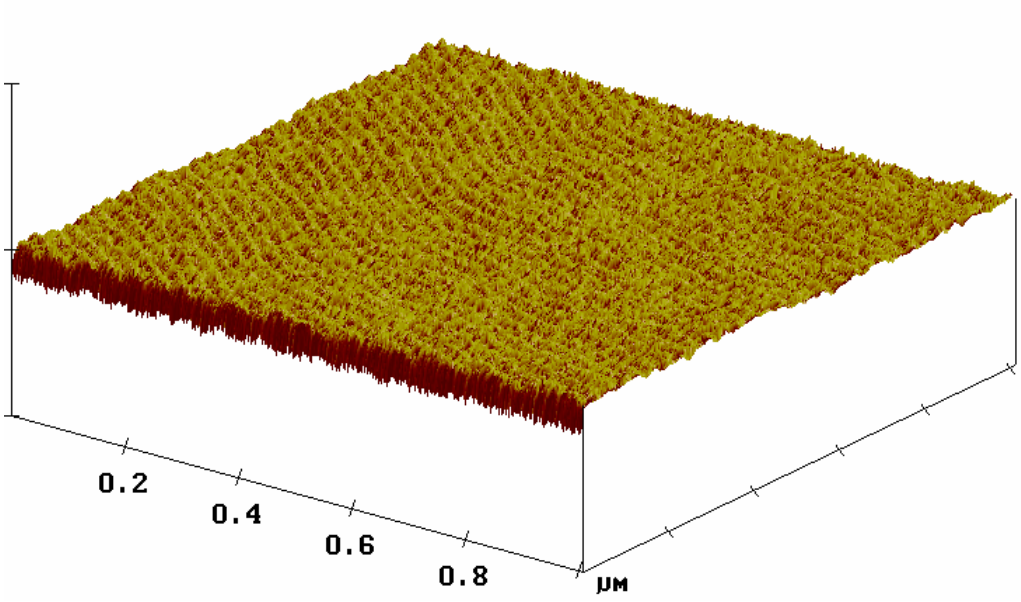


Figure 12: AFM Images of Carbon (1.00 X 1.00 [µm] Z-Max 10.0 nm)

5.1.3. X-Ray Diffraction images of Carbon

X-Ray diffraction analysis was done to evaluate the structure of carbon substrate. The curve indicates that the carbon has a graphitic structure making it ideal to be used for electrode purposes.

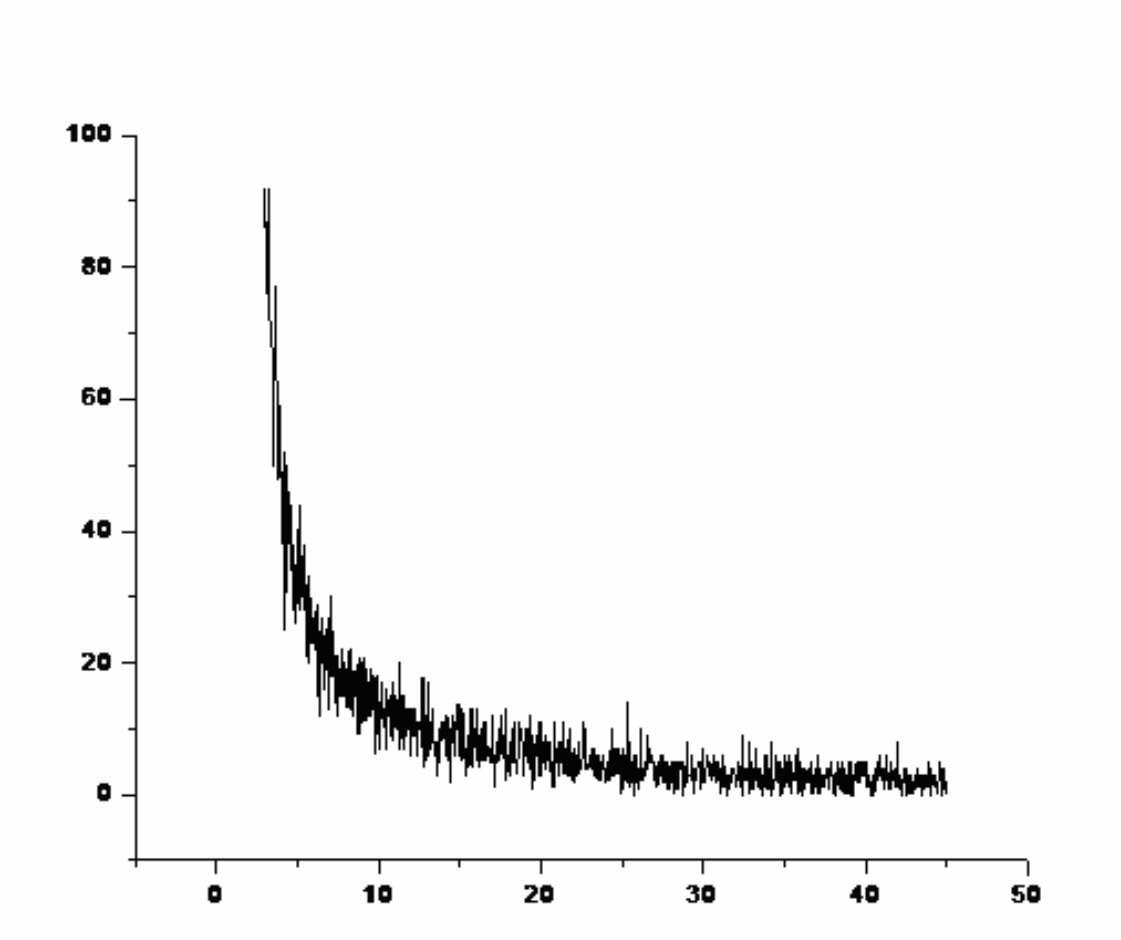


Figure 13: X-Ray Diffraction Analysis of the Carbon substrate

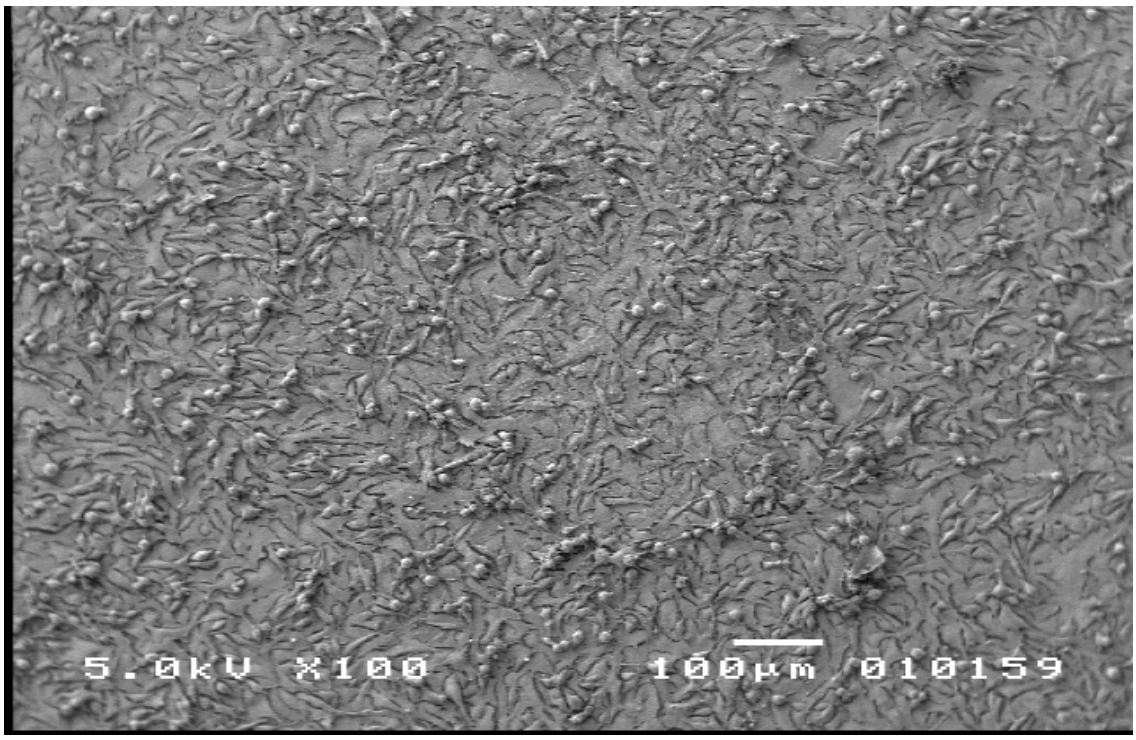
5.2. Cell Culture on Carbon

5.2.1. SK-N-MC cells on Carbon Substrate

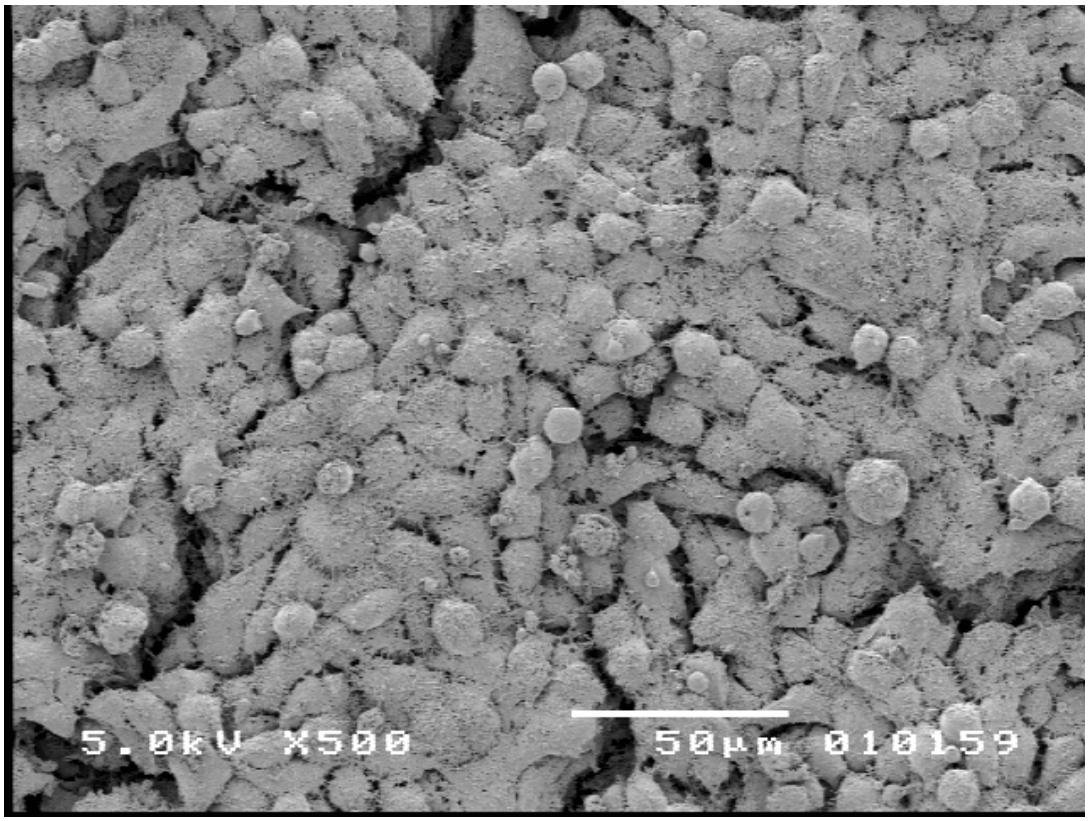
Human neuroblastoma, SK-N-MC cells exhibit excellent adhesion even on untreated surfaces. Fig. 14(a,b,c) presents the scanning electron microscope images of neuroblastoma cells cultured on the carbon template at a concentration of 3 ×105 cells/ml. Within 2 days of cell culturing, the surface of the template was observed to be covered entirely with cells. After 4 days of culturing cells started growing on top each other, cell growth rate was high Fig. 15 .Fig. 16 represents cell growth after a week.

Figure 14: SEM Images of Human Neuroblastoma of SK-N-MC cells – Observations after 2 days

(a) At a magnification of 100 X



(b) At a magnification of 500X , SK-N-MC cells are seen growing well and in large numbers on the photoresist derived carbon substrate after 2 days



(c) At a magnification of 1000 X , the illustration shows the structure of each cell growing on carbon

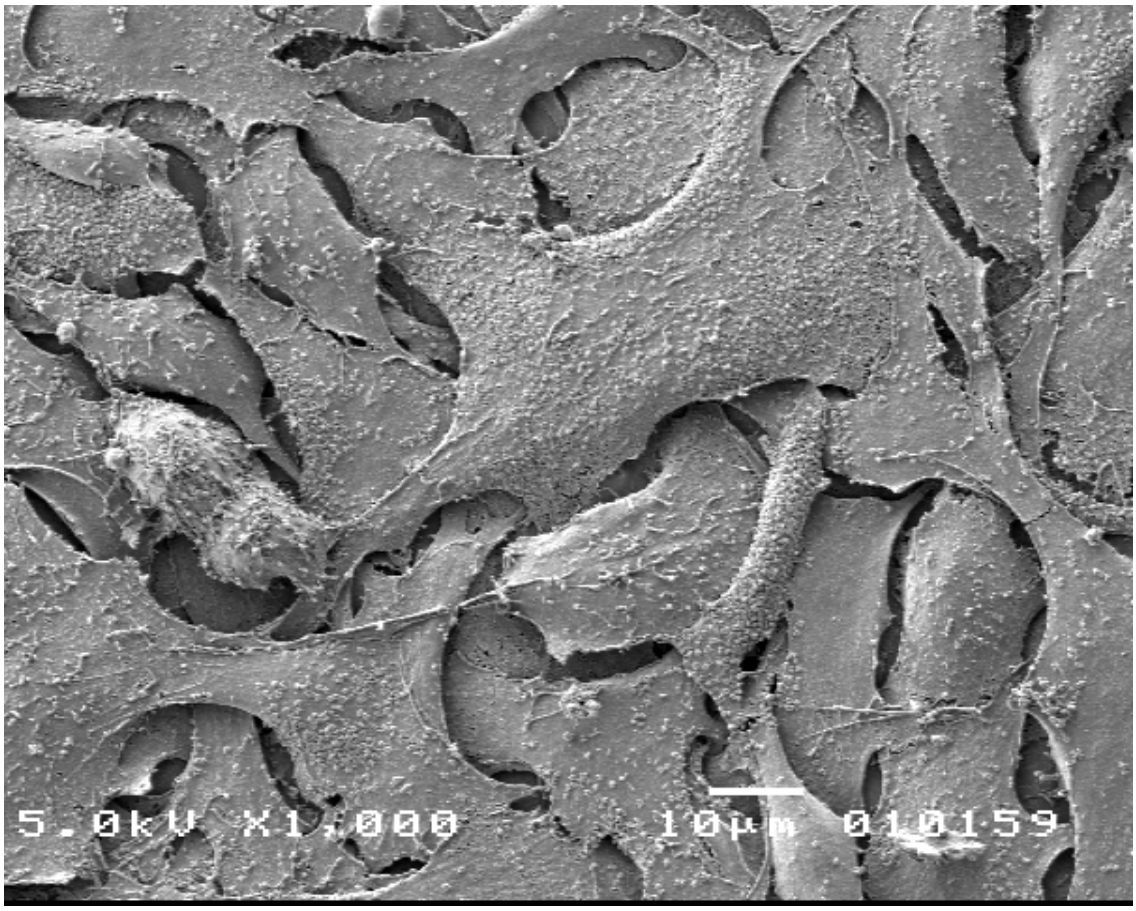
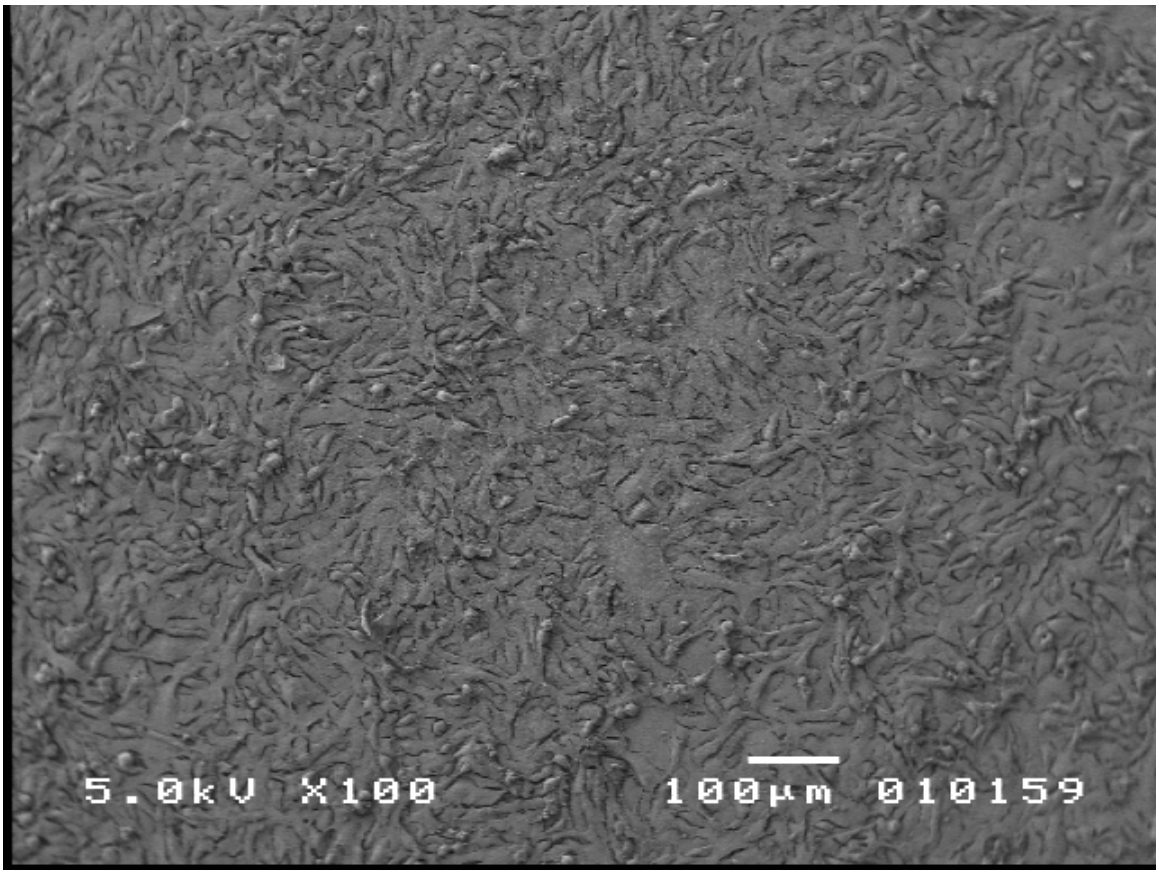
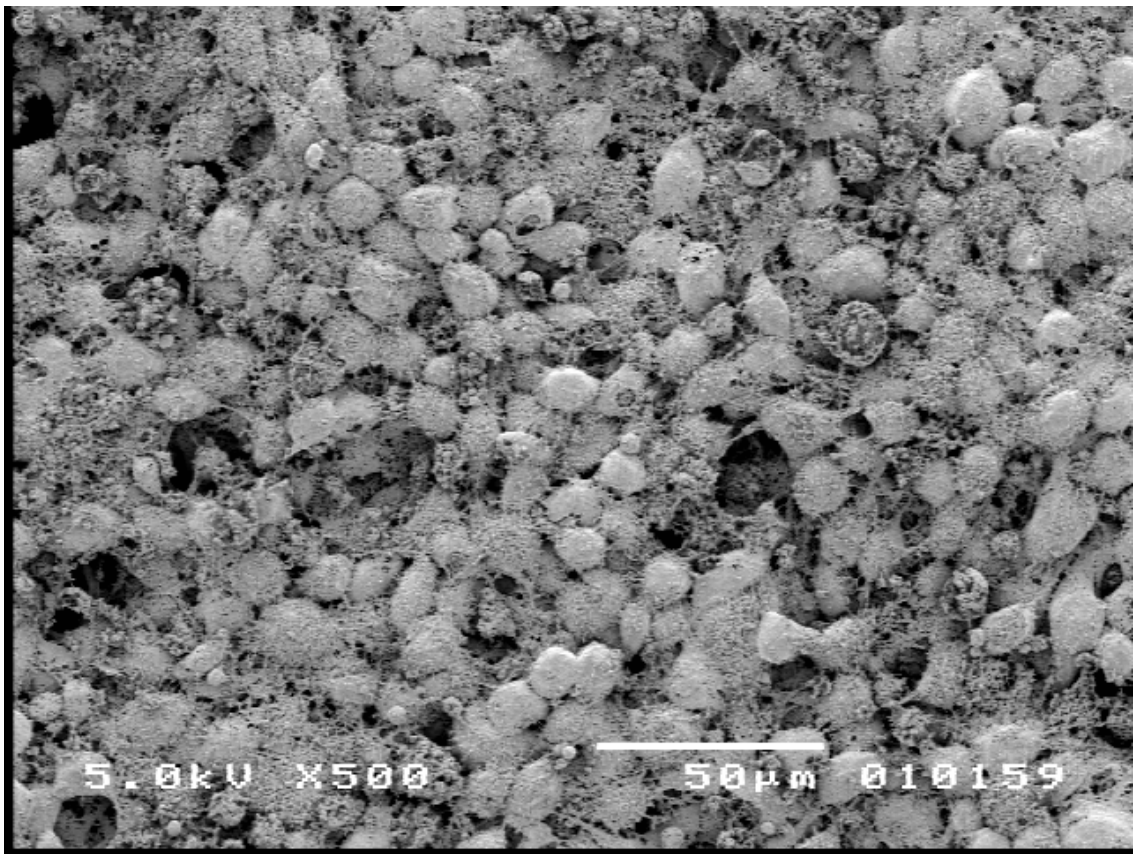


Figure 15: SEM Images of Human Neuroblastoma of SK-N-MC cells – Observations after 4 days

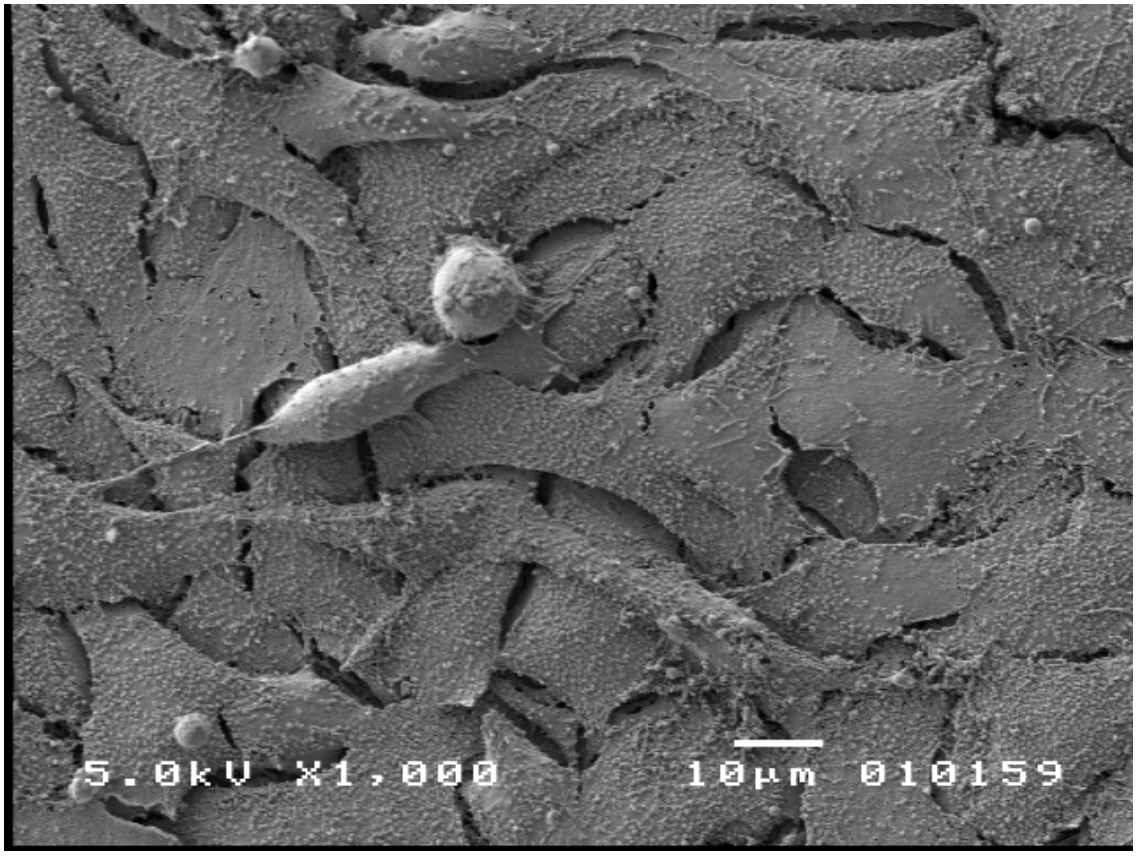
(a) At a magnification of 100X



(b) At a magnification of 500X



(c) At a magnification of 1000X



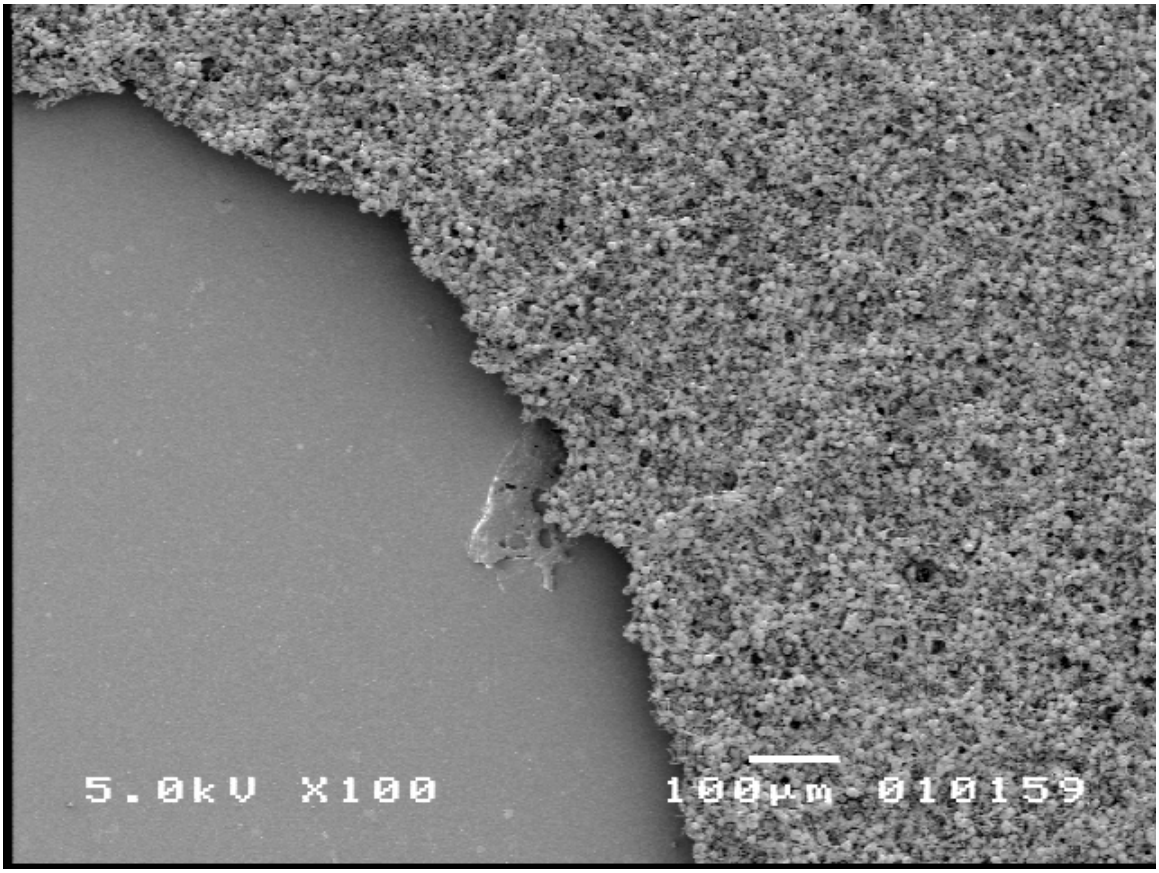
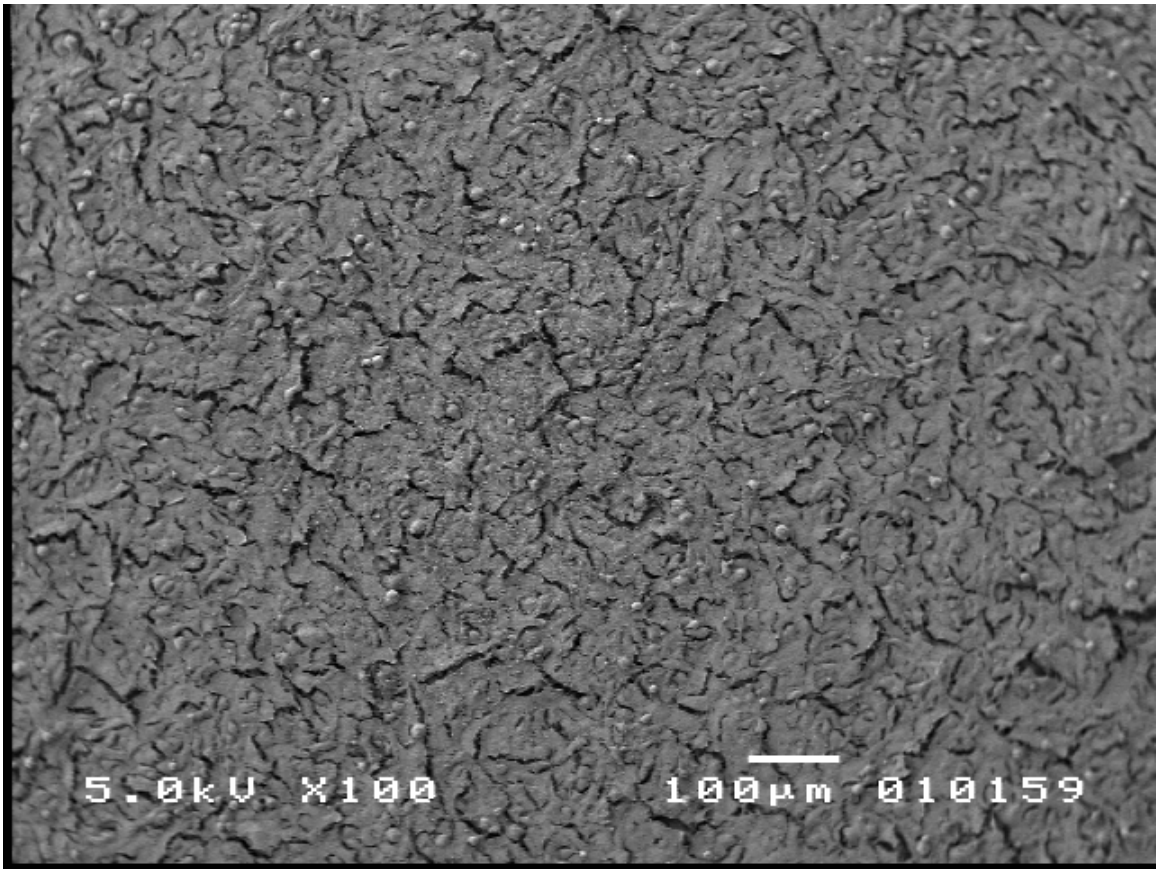


Figure 16: SEM Images of Human Neuroblastoma of SK-N-MC cells - Observation after a week

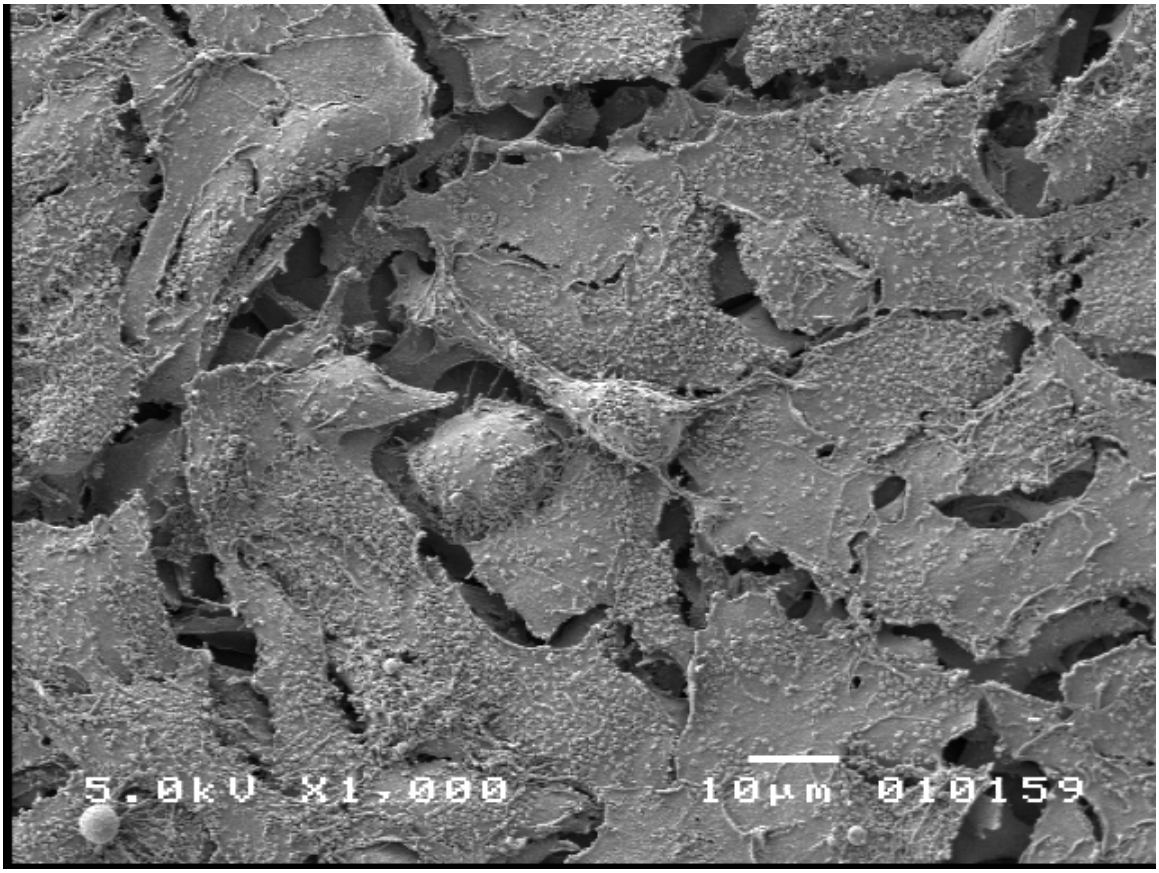
5.2.2. SY5Y cells on Carbon Substrate

Another type of human neuroblastoma cells-SY5Y was also tested to confirm the affinity of neuroblastoma cells to unfunctionalized photoresist derived carbon. Fig.17 (a,b) presents the growth and proliferation of SY5Y cells cultured on carbon template and observed for 2 days and Fig. 18 (a,b) represents growth after 4 days.

Figure 17: SEM Images of Human Neuroblastoma of SY5Y cells on Carbon substrate (after 2 days)

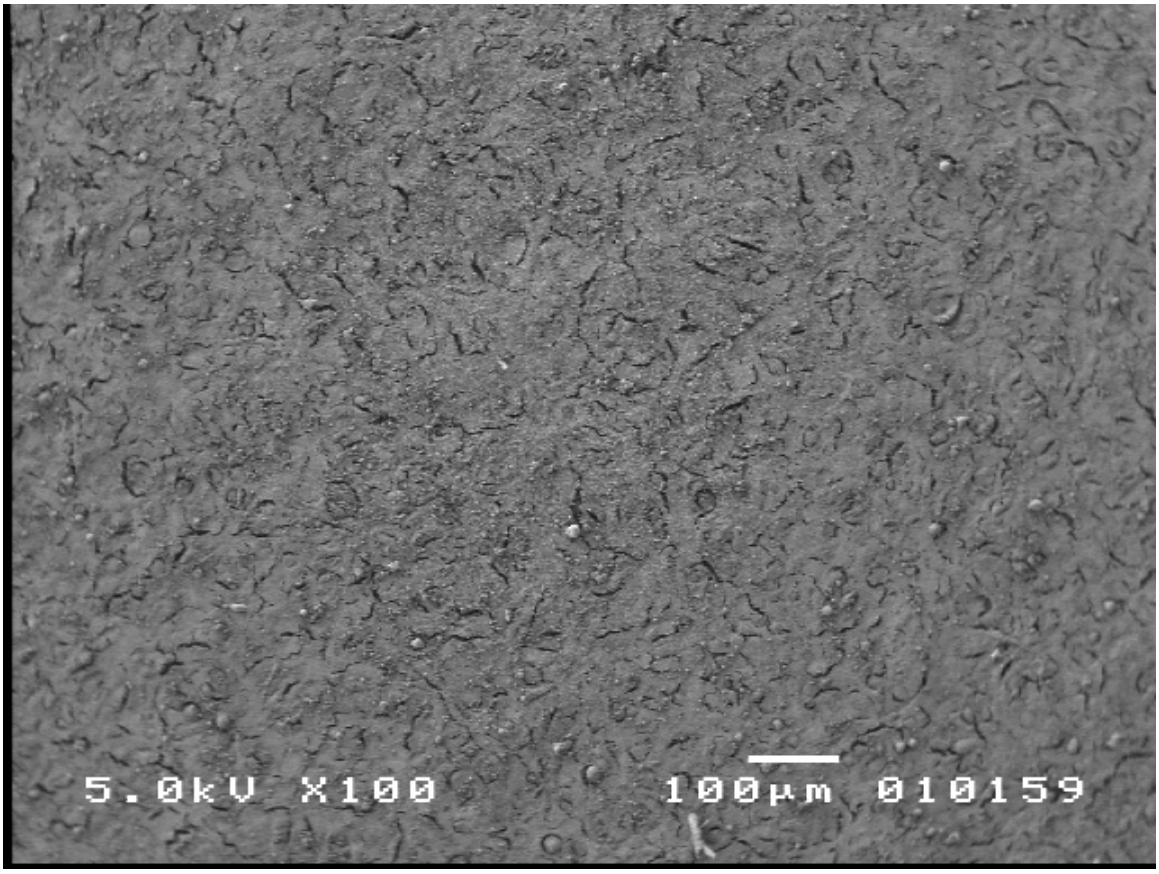


(a) At a magnification of 100X

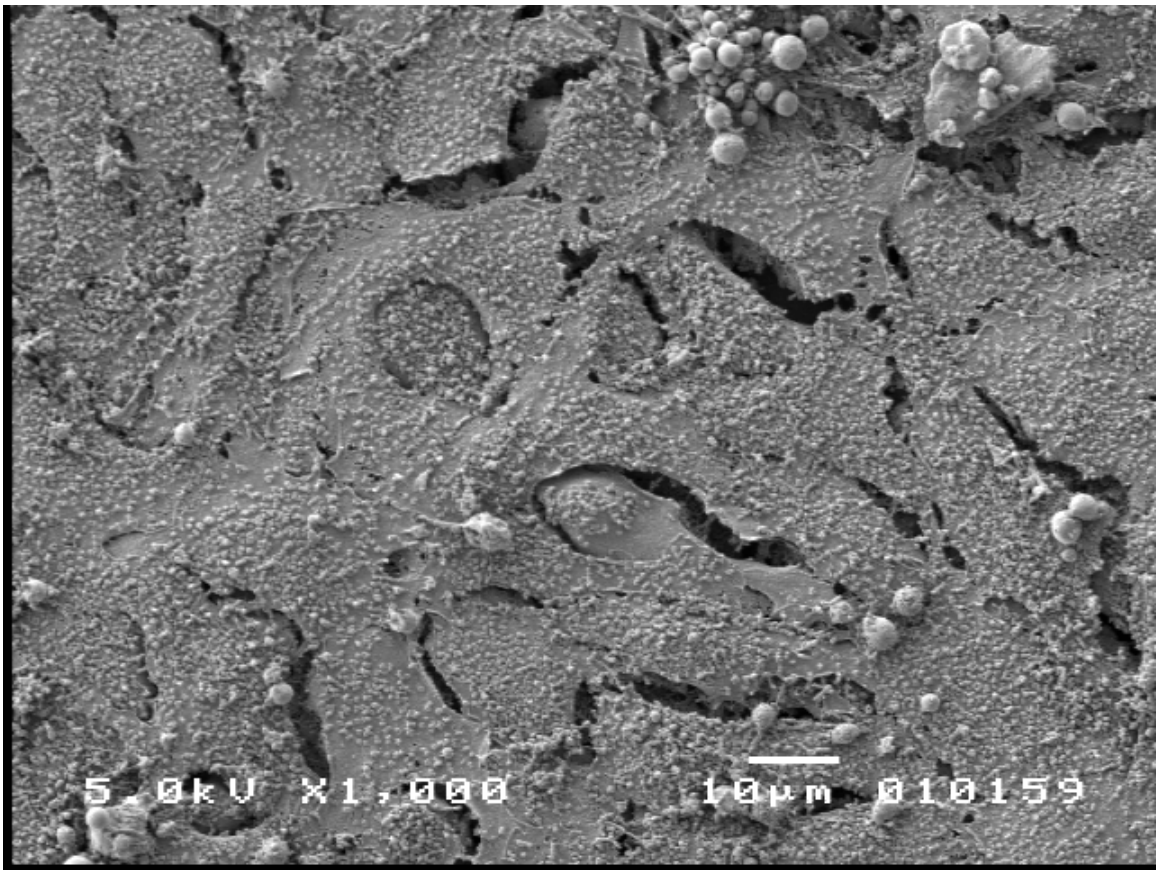


(b) At a magnification of 1000X

Figure 18: SEM Images of Human Neuroblastoma of SY5Y cells on Carbon substrate (after 4 days)



(a) At a magnification of 100X

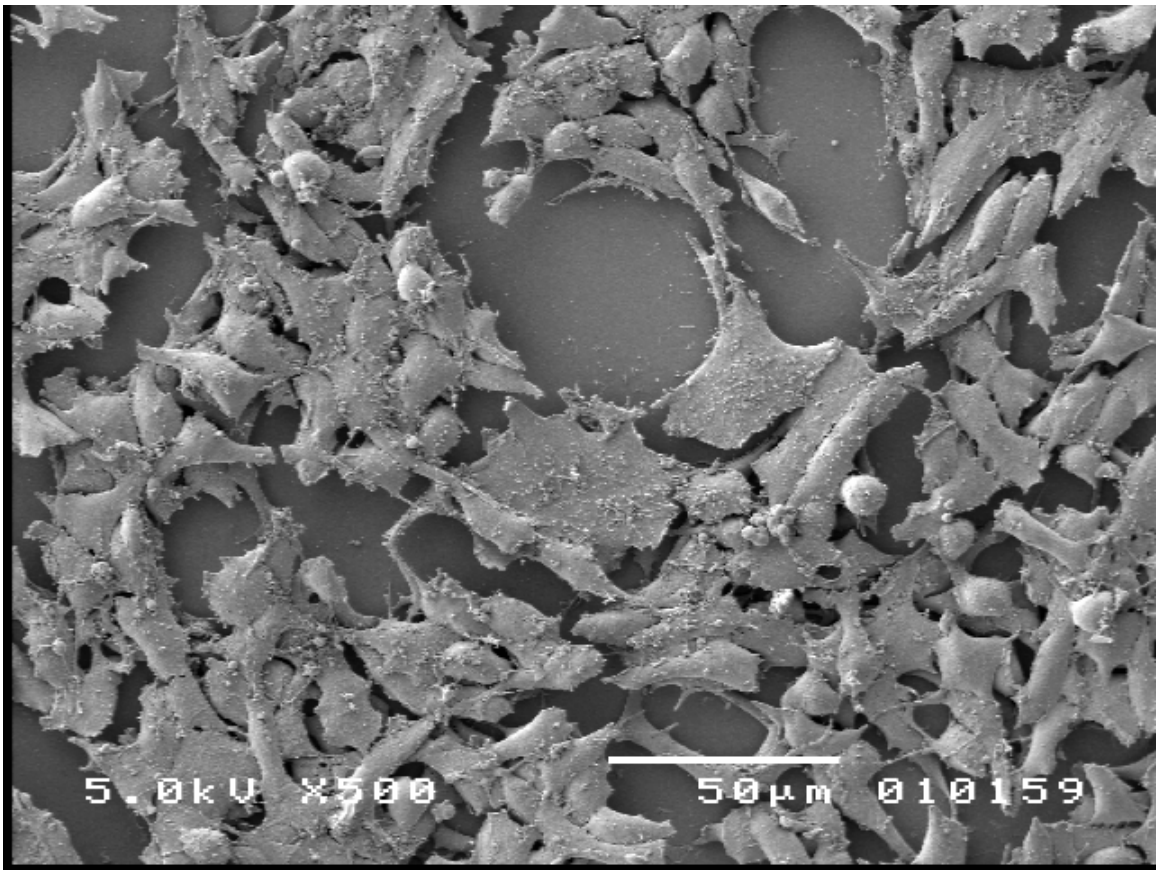


(b) At a magnification of 1000X

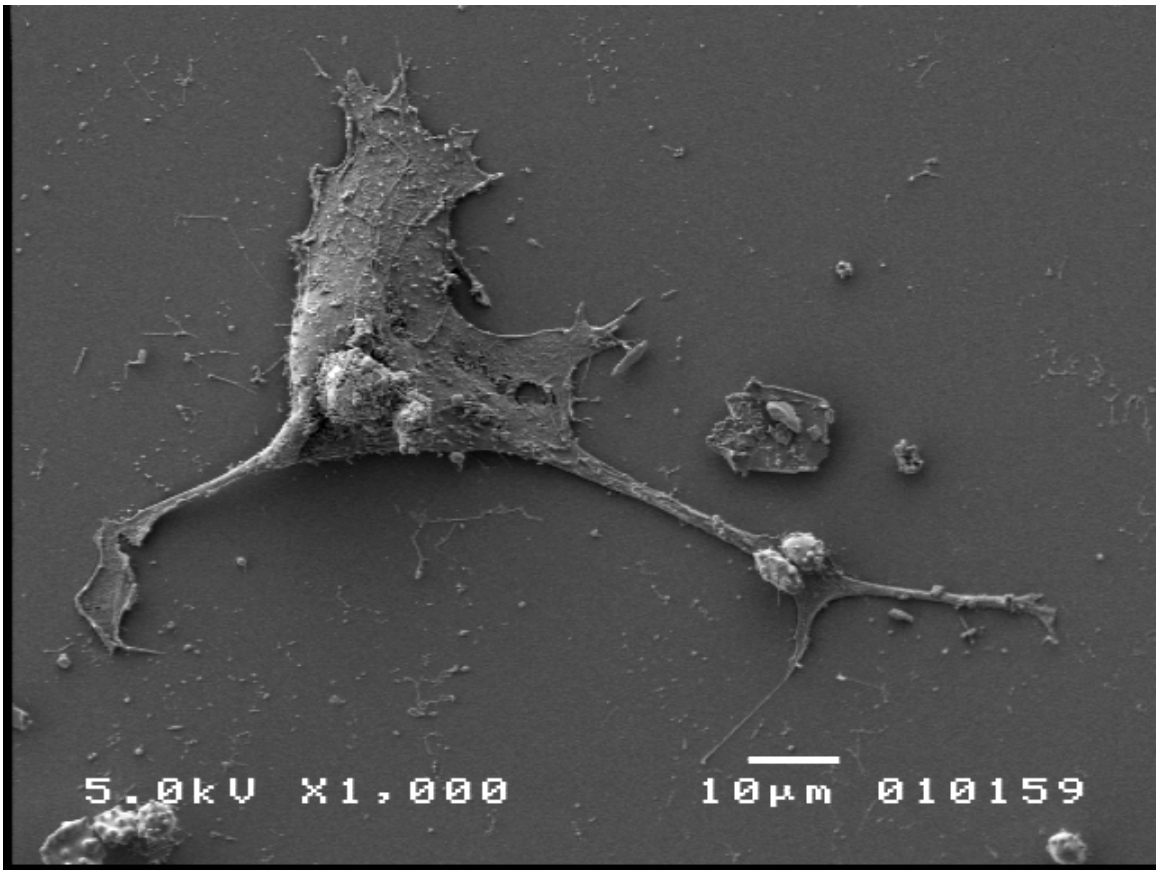
5.2.3. P-19 cells suspended on Carbon Substrate

We also investigated mouse teratocarcinoma – P19 cells, SEM images represent the scanning electron microscope images of P19 cells fixed after 3 days Fig. 19. Cells were grown at a concentration of 2×10^4 cells/ml to avoid excessive populating of cells, as P19 cells are known to grow faster, absorbing all the nutrients from cell culture medium, leaving the medium acidic, resulting in early cell death.

Figure 19: SEM Images of P-19 cells on Carbon substrate



(a) At a magnification of 500X



(b) At a magnification of 1000X

5.3. PC 12 Cell Differentiation

Scanning electron micrographs of PC12 cells differentiation observed at the 1 (Fig 20), 2 (Fig 21), 3 (Fig 22) and 4 day (Fig 23) time points are shown below. Neurite outgrowth was observed after 24 hours of nerve growth factor injection. Cells closer to each other started forming synapses after a day. Cells tend to become less adhesive due to the growing tension in the neurite that connects the cells. This tensile force dominates the adhesion force causing less lose contact with the substrate. However, cells grown on photoresist derived carbon remained on the substrate even after four days and extending to more than hundred microns to reach to the distant cells.

Figure 20: PC-12 Cell Differentiations – After 24 hours

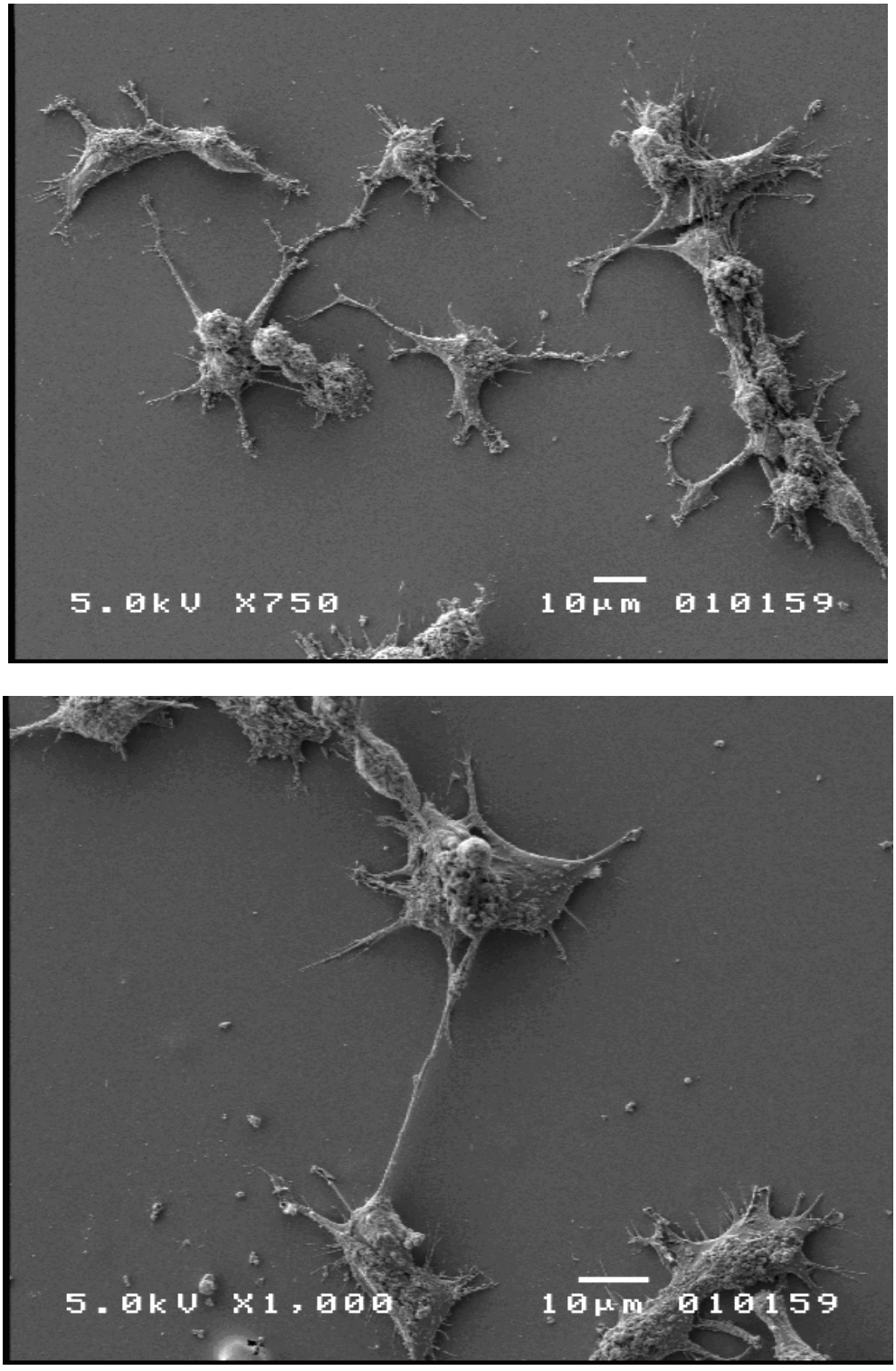


Figure 21: PC-12 Cell Differentiation – 2 days after NGF induction

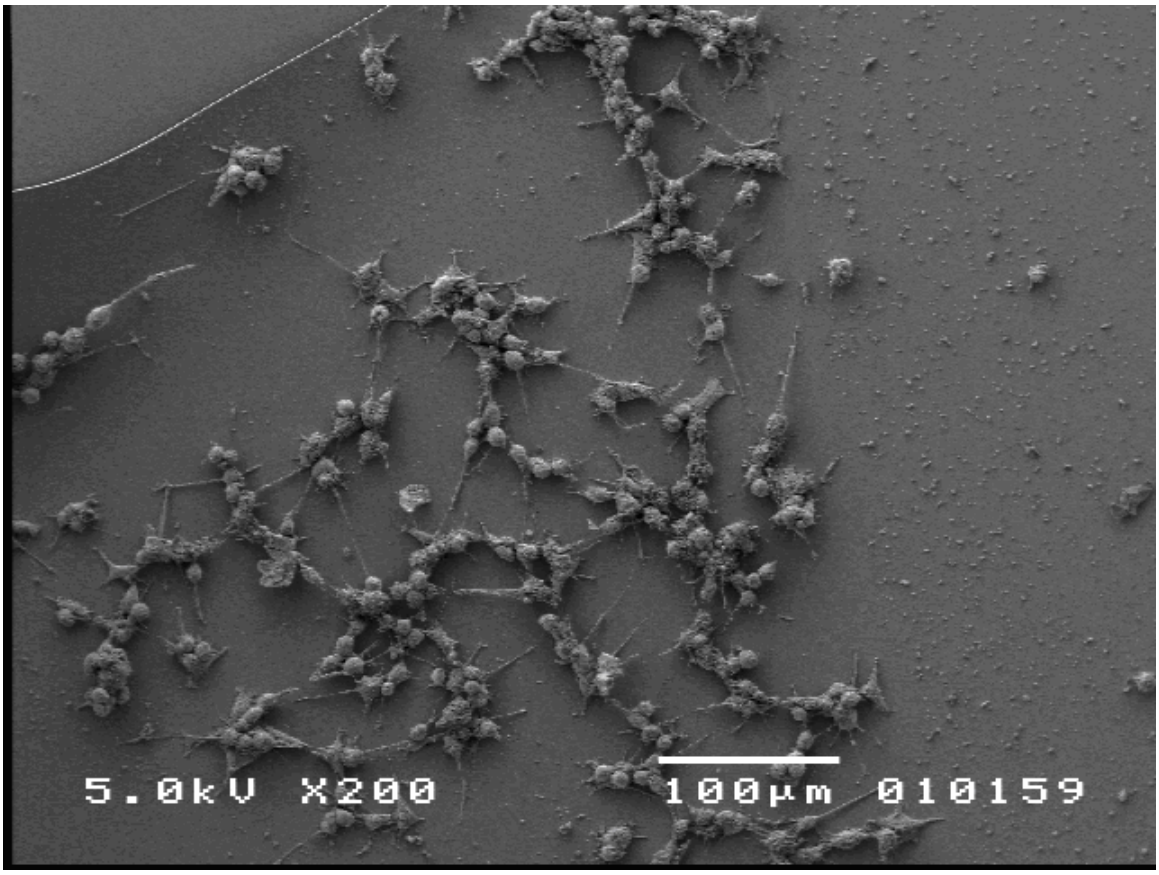
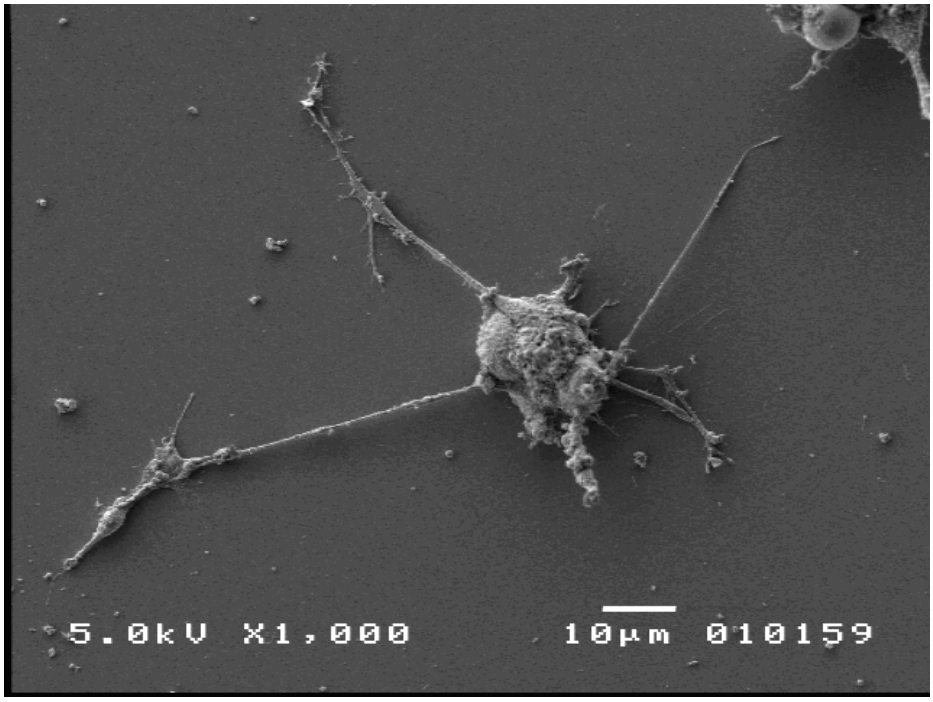


Figure 22: PC-12 Cell Differentiations – After 3 days



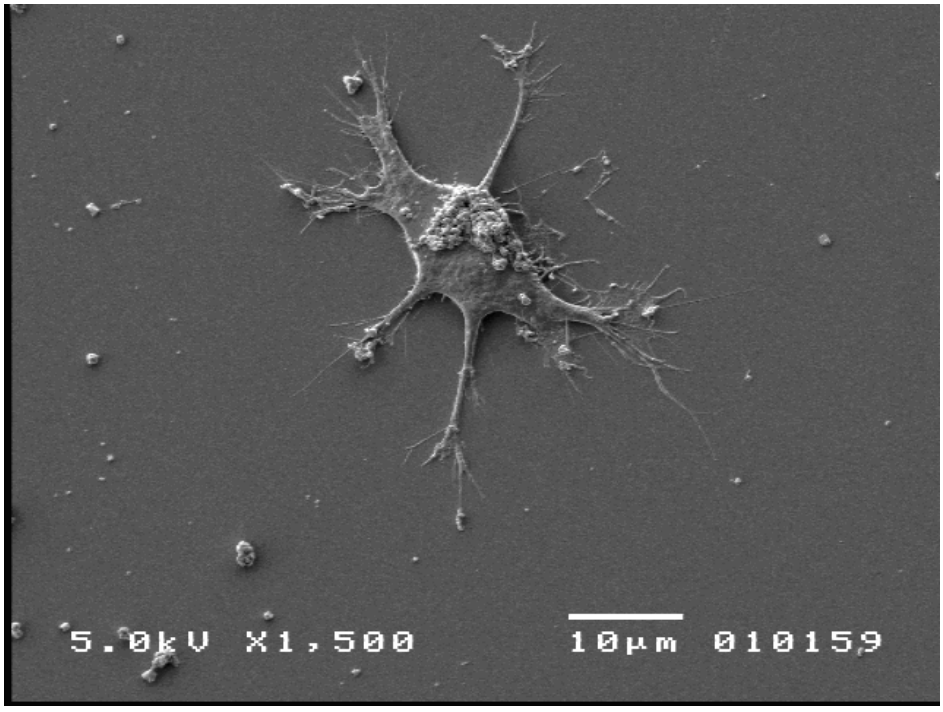
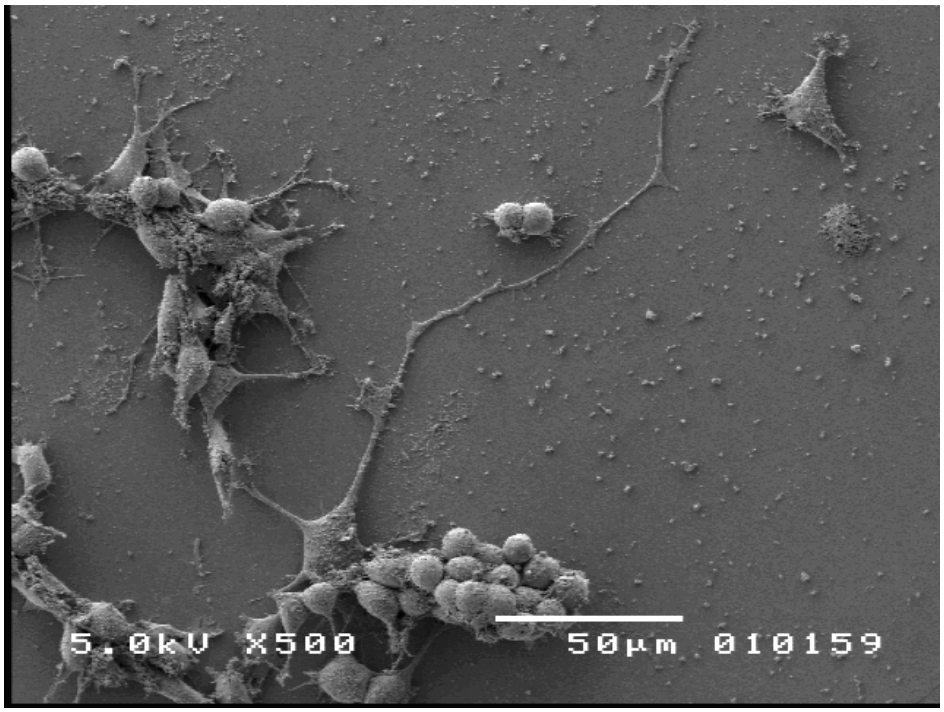
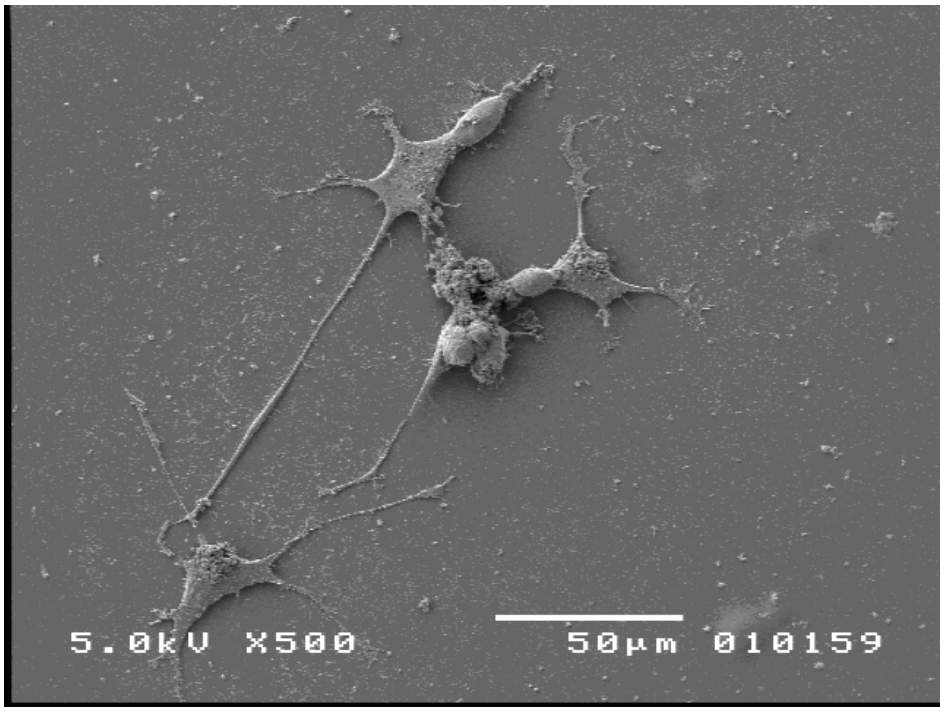


Figure 23: PC-12 Cell Differentiations – After 4 days of NGF induction





Differences in neurite growth were quantified by measuring neurite length (Fig 22), the number of neuritis emanating per cell (Table 2). Neurites emerging from cells grown on carbon were significantly longer than neurites emerging from control cells as shown on the plot.

Table 2: Average Values for measurements of Neurite Growth for differentiated PC 12 cells, cultured on Photoresist derived carbon and Poly-l-Lysine coated glass (control)

	Photoresist derived carbon		Poly-L-lysine coated glass	
Time period	L_{Neurite} (μm)	N_{Neurite} (per cell)	L_{Neurite} (μm)	N_{Neurite} (per cell)
Day 1	20 ± 10	2 ± 2	15 ± 10	1 ± 2
Day 2	40 ± 10	5 ± 2	30 ± 10	2 ± 2
Day 3	50 ± 10	6 ± 2	40 ± 10	4 ± 2
Day 4	100 ± 10	6 ± 2	60 ± 10	5 ± 2

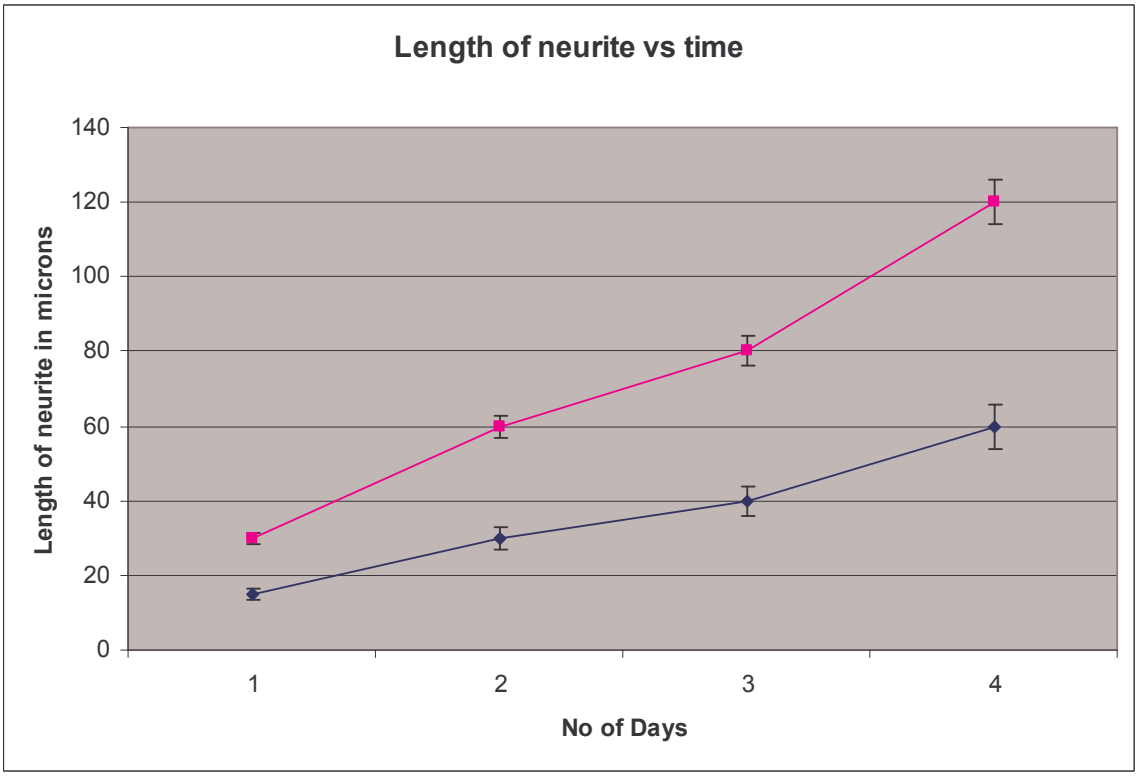


Figure 24: Length of Neurite as a function of time

5.4. Gel Electrophorosis Analysis

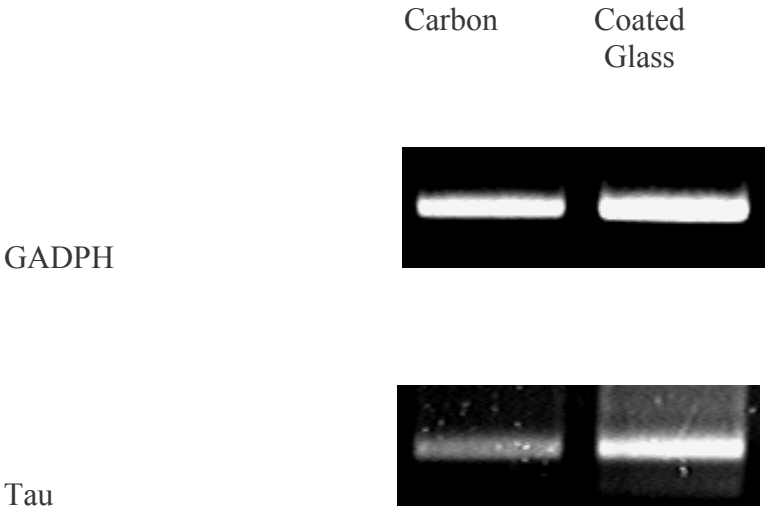


Figure 25: mRNA gene expression of GADPH and Tau in PC-12 cells

Gel electrophoresis images show the bands of GADPH and Tau gene bands on agarose gel. As seen, there is no difference in the expression of bands confirming the normal growth of cells on photoresist derived carbon substrate.

6. CONCLUSION

Neural implants have been fabricated using a variety of natural and synthetic materials to create a stable environment for the regeneration of nerves. The templates provide a support structure and stimulate injured nerves to restore neuronal signaling pathways. The challenge of nerve tissue engineering lies in producing a guide that is biocompatible, biodegradable, and easy to use in surgical applications. Currently either carbon nanotubes [7,8,9], silicone [5,6] or biopolymers or naturally occurring proteins [3,4] have fulfilled all the attributes.

We investigated photoresist derived carbon substrate to be used as a template for regeneration of nerves. We used SPR 220.7 positive photoresist as a precursor to generate carbon. The photoresist was spin coated on silicon water, the process involved four applications of photoresist , spun at 300 rpm for 3 seconds and then spun at 3000 rpm for 30 seconds. Silicon wafer with coated photoresist was soft baked at 95ºC for 5 minutes. Postbaking, photoresist was pyrolyzed in a close tube furnace in nitrogen environment. Before the start of the heating cycle the furnace atmosphere was made inert by flowing gas for 10 minutes.

Various human and rat neurons were cultured on sterilized carbon substrate. We investigated affinity of human neuroblastoma- SK-N-MC and SY5Y cells. Rat teratocarcinoma cells were also tested for its growth and survival on the carbon film.

We also obsereved neurite outgrowth of rat pheochromocytoma –PC 12 cells when injected with nerve growth factor. The length and number of neurtires emerging from each cell soma were compared with the cells grown on coated glass cover slips .

In order to complete the characterization, the cells were tested at their molecular level. We employed reverse transcription polymerase chain reaction technique to amplify the mRNA extracted from the cells cultured on carbon and on coated glass slip. We used gel electrophoresis technique to observe the expression of GADPH and Tau gene. Thus, photoresist derived carbon can be a promising template for regeneration of neurons. Being electrically conductive it can also be used to study the electrophysiology of neurons.

7. FUTURE WORK

Topography of the substrate plays a vital role in determining cell response of cells to a structure. We can take the advantage of using photoresists as the starting material for carbon template, as the photoresists can be patterned by photolithography techniques.. Once cells have been plated onto carbon structure in a specific pattern, we can selectively eliminate cells from the established network and study how cells reconnect after injury.

The ability to grow cultured neurons in a specific pattern and be able to record electrical activity will open up the area of study in neuronal networks and function. It would be then possible to record the extracellular potential from a cell body or the firing of an individual fiber to the outstanding electrical properties of photoresist derived carbon.

8. References

1. R.D.Evans, G., *Challenges to Nerve Regeneration.* Seminars in Surgical Oncology, 2000. **19**: p. 312-318.
2. Hubbell, J.A., *Bioactive Biomaterials.* Current Opinion in Biotechnology, 1999. **10**: p. 123-129.
3. Massia SP, H.J., *An RGD spacing of 440 nm is sufficient for integrin $\alpha_{\varpi}\beta_{3}$– mediated fibroblast spreading and 140 nm for local contact and stress fiber formation.* J Cell Biol, 1991. **114**: p. 1089-1100.
4. Massia SP, R.S., Hubbell JA, *Covalently immobilizd laminin peptide Tyr-lle-Gly-Ser-Arg (YIGSR) supports cell spreading and co-localization of the 67-kilodalton laminin receptor with a-actinin and vinculin.* J BioL Chem 1993. **268**: p. 8053-8059.
5. Y.S.Chen, C.L.H., .C. Tsai, T.H.Chen, W.C.Cheng, C.L.Hu,C.H. Yao, *Peripheral Nerve Regeneration using Silicone Rubber Chambers Filled with Collagen, Laminin and Fibronectin.* Biomaterials, 2000. **21**: p. 1541.
6. Fromherz, P., *Electrical Interfacing of Nerve Cells and Semiconductore Chips.* ChemPhyChem, 2002. **3**: p. 276-284.
7. Thomas J Webster, M.C.W., Jaince L McKenzie, Rachel L Price and Jeremiah U Ejiofor, *Nano-biotechnology:carbon nanofibers as improved neural nd orthopaedic implants.* Nanotechnology, 2004. **15**: p. 48-54.

8. Xuan Zhang, S.P., Sandip Niyogi, Andre Morgan, Mihri Ozkan, Cengiz S. Ozkan, *Guided neurite growth on patterened carbon nanotubes.* Sensors and Actuators B, 2005. **106**: p. 843-850.

9. J.C.Bokros, *Carbon in Medical Devices.* Ceramics International, 1983. **9**(3-).

10. Jenkins, G.M., *Biomedical Applications of Carbons and Graphites.* Clin.Phys.Physiol.Meas, 1980. **1**(3): p. 171-194.

11. J.Kim, X.S., K.Kinoshita, M.Madou, and R.White, *Electrochemical Studies of Carbon Films fom Pyrolyzed Photoresist.* Journal of the Electrochemical Society, 1998. **145**(7).

12. Chunlei Wang, L.T., Guangyao Jia, Marc Madou, Yuting Yeh and Bruce Dunn, *C-MEMS for the Manufacture of 3D Microbatteries.* Electrochemical and Solid-State Letters, 2004. **7**(11): p. A435-438.

13. Chih-Jung Yao, G.-M.L., Chin-Feng Chan, Ya-Yu Yang, Frank C.Liu and Shuang-En Chuang, *Differentiation of Pheochromocytoma PC 12 Cells Induced by Human Urine Extract and the Involvement of the Extracllular Signal-Regulated Kinase Signaling Pathway.* The Journal of Alternative and Complementary Medicine, 2005. **11**(5): p. 903-908.

14. Akio Kishida, S.K., Kaori Ohmura, Kazuhisa Sugimura and Mitsuru Akashi, *Evaluation of biological responses to polymeric biomaterials by RT-PCR analysis:Study of IL-1b mRNA expression.* Biomaterials, 1996. **17**(13): p. 1301-1305.

15. Fawcett JW, A.R., *The glial scar and central nervous system repair.* Brain Res Bull, 1999. **49**: p. 377-391.

16. Bessea L., C.B., Lebreton-Decoster C, Giraud-Guille MM, *Production of ordered collagen matrices for three-dimensional cell culture.* Biomaterials, 2002. **23**: p. 27-36.

17. Labrador RO, B.M., Navarro X., *Influence of collagen and laminin gels concentration on nerve regeneration after resection and tube repair* Exp Neurol, 1998. **149**(243-252).

18. Large TH, B.S., Clegg DO, Weskamp G, Otten U,, *Nerve growth factor gene expression in the developing rat brain.* Science, 1986. **234**(352-355).

19. Renauddin A, L.M., Girault J, McKerracher L., *Organization of point contacts in neuronal growth cones.* J Neurosci Res, 1999. **55**: p. 458-471.

20. Patrick Jr. CW, M.A., McIntire lv, , *Frontiers in tissue engineering.* Oxford Pegamon, 1998: p. 3-11.

21. Langer R, V.J., *Tissue engineering.* Science, 1993. **260**(5110): p. 920-926.

22. M.Borkenhagen, J.F.C., H. Sigrist, P. Aebischer, *Three-dimensional extracellular matrix engineering in the nervous system* J Biomed Mater Res, 1998. **40**: p. 392-400.

23. den Dunnen WF, M.M., Grrijpma DW, Robinson PH, Schakenraad JM, *In vivo and in vitro degradation of poly/D/CL and the implictions for the use in nerve reconstruction.* J Biomed Mater Res, 2000. **In vivo and in vitro degradation of poly/D/CL and the implictions for the use in nerve reconstruction**(51): p. 575-585.

24. Doolabh VB, H.M., Mackinnon SE, *The role of conduits in nerve repair.* Rev Neurosci, 1996. **7**(47-84).

25. Rutkowski GE, H.C., *Development of a bioaftificial nerve graft* Biotechnol Prog, 2002. **18**: p. 373-379.

26. Brewer GJ, D.S., Ponnusamy E., *Precocious axons and improved survival of rat hoppicampal nurons on lysine-alanine polymer substrates.* J Neurosc Methods, 1998. **85**: p. 13-20.

27. Buettner HM, P.R., *Quantitative effects of laminin concentration on neurite outgrowth in vitro.* Dev Biol

1991. **145**: p. 266-276.

28. Keihoff G., S.F., Wolf G, Fansa H., *Biocompatibiity of type I/III collagen matix for peripheral nerve reconstruction.* Biomaterials, 2003. **24**: p. 2779-2787.

29. Archibald SJ, K.C., Shener J, Li ST, Madison RD, *A collagn based nerve guide conduit for peripheral nerve repair: an electrophysiological study of nerve regenertion in rodents and nonhuman primates.* J Comp Neurol, 1991. **306**(685-696).

30. LR, W., *Exognous finbrin matrix precursors stimulate the temporal [rogress of nerve regeneration within a silicone chamber.* Neurochem Res, 1987. **12**: p. 851-860.

31. Whitworth IH, B.R., Dore CJ, Anand P, Green CJ, *Nerve growth factor enhances nerve regeneration through fibronectin grafts.* J Hand Surg, 1996. **21**: p. 514-522.

32. Chafik D, B.D., Bui P, *Optimization of schwann cell dhesion in response to shear stress in an vitro model for peripheral nerve tissue engineering.* Tissue Emg, 2003. **9**: p. 233-241.

33. Gues, K.P.D.J.a.J.W., *Carbon nanofibers: Catalytic synthesis and applications.* Catal Rev. -Sci Eng., 2000. **42**(4): p. 481-510.

34. Benjamin S. Harrison, A.A., *Carbon Nanotube Applications for Tissue Engineering.* Biomaterials, 2007. **28**: p. 344-353.

35. Rachel L. Price, K.E., Karen M.Haberstroh, Thomas J. Webster, *Nanometer surface roughness increases select osteoblast adhesion on carbon nanofiber compacts.* J Biomed Mater Res, 2004. **70A**: p. 129-138.

36. Tamir Gbay, E.J., Eshel Ben-Jacob, *Engineered self-organization of neural networks using carbon nanotube clusters.* Physica A, 2005. **350**: p. 611-621.

37. Amit Singh, J.J., Marc Madou and Sheikh Akbar, *Pyrolysis of negative phtoresist to fabricate cabon structures for microelectromechanical systems and electrochemical application.* Journal of the Electrochemical Society Procedings, 2002. **97**(19): p. 61.

38. Srikanth Ranganathan, R.M., Sree Mouli Majji and Marc Madou *Photoresist - Derived Carbon for Microelectromechanical System and Electrohemical Applications.* Journal of the Electrochemical Society 2000. **147**(1): p. 277-282.

39. Miller C, J.S., Mallapragada S, *Synergistic effects of physical and chemical guidance cues on neurite alignment and outgrowth on biodegradable polymer substrates.* Tissue Emg, 2002. **8**: p. 367-378.

40. Clark P, B.S., Connolly P., *Growth cone guidance and neuron morphology on micropatterend laminin surfaces.* J Cell Sci, 1993. **105**: p. 203-212.

41. Miller C, J.S., Mallapragada S, *Micropatterned schwann cell seeded biodegrdabale polymer substrates significantlly enhance neurite alignment and outgrowth.* Tissue Emg, 2001. **7**: p. 705-715.

42. den Braber ET, d.R.J., Ginsel LA, von Recum AF, *Orientation of ECM protein deposition, fibroblast cytoskeleton, and attachment complex components on silicone microgrooved surfaces.* J Biomed Mater Res, 1998. **40**(291-300).

43. Kam L, S.W., Turner JN, Bizios R, *Axonal outgrowth of hippocampal neurons on micro-scale networks of polylysine-conugated laminin.* Biomaterials, 2001. **22**: p. 1049-104.

44. William M.Freeman, S.J.W.a.K.E.V., *Quantitative RT-PCR: Pitflls and Potential* BioTechniques, 1999. **26**: p. 112-125.

45. M., H.X.a.R., *Mechanisms of complex transcriptional regulation:Implications for brain development.* Neuron, 1991. **7**: p. 183-196.

46. Himmler, A., *Structur of a bovine tau gene: alternatively spliced transcripts generate a protein family.* Mol Cell Biol, 1989. **9**: p. 1389-1396.

47. Binder L, Frankfurter A. and Rebhun L., *The distribution of tau in the mammalian central nervous system.* J Cell Biol, 1985. **101**: p. 1371-1378.

48. Drubin D.G, K., S. and Kirschner M, *Association of tau protein with microtubules in living cells.* Dynamic Aspects of Molecular Biology, 1986. **466**: p. 257-268.

49. Harada. A., O.K., Okabe S., Kuno J, *Altered microtubule organization in small-calibre axons of micelacking tau protein.* Nature, 1994. **369**: p. 488-491.

50. K., K., *Tau protein and neurodegeneration.* Mol Neurobiol, 1992. **4**: p. 171-179.

51. Andrealis A., B., W.and Kosik K., *Structure and novel exons of the human tau gene.* Biochemistry, 1992. **31**: p. 10626-10633.

52. Sadot E., M.R., Barg J., Behar L. and *Complete sequence of 3"- untranslated region of tau from rat central nervous system.* J Mol Biol, 1994. **241**: p. 325-331.