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IZMIR KATIP CELEBI UNIVERSITY

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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

EVALUATION OF NEURAL DIFFERENTIATION OF PC12 CELLS GROWN ON GRAPHENE COATED ITO MICROCHIPS

M.Sc. THESIS

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İZMİR KATİP CELEBİ ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

GRAFEN KAPLANMIŞ ITO MİKROÇİP ÜZERİNDE BÜYÜTÜLEN PC12 HÜCRELERİNİN NÖRONAL FARKLILAŞMASININ DEĞERLENDİRİLMESİ

YÜKSEK LİSANS TEZİ

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Tansu GÖLCEZ a **M.Sc.** student of **IKCU Graduate School of Natural And Applied Sciences**, successfully defended the thesis entitled "**EVALUATION OF NEURAL DIFFERENTIATION OF PC12 CELLS GROWN ON GRAPHENE COATED ITO MICROCHIPS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family

FOREWORD

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ABBREVIATIONS

CNS: Central Nervous System

ANS: Autonomic Nervous System

SNS: Sympathetic Nervous System

PNS: Parasympathetic Nervous System

ITO: Indium Tin Oxide

ACh: Acetylcholine

CE: Capillary Electrophoresis

BBB: Blood Brain Barrier

NPs: Nanoparticles

OPCs: Oligodendroglial Precursor Cells

LFES: Low-Frequency Electrical Stimulation

ES: Electrical Stimulation

BDNF: Brain-Derived Neurotrophic Factor

LOC: Lab-on-a-chip

EC: Electrochemical

EKs: Electrokinetics

PDMS: Polydimethyl Siloxane

SU-8: Epoxy Resins

UV: Ultra Violet

MEMS: Microelectromechanical Systems

3D: 3-Dimensional

RE: Reference electrode

CE: Counter Electrode

WE: Working Electrode

SPR: Surface Plasmon Resonance

QCM: Quartz Crystal Microbalance

GO: Graphene Oxide

rGO: Reduced Graphene Oxide

ITO: Indium Tin Oxide

NGF: Nerve Growth Factor

DMSO: Dimethyl Sulfoxide

DHS: Donor Horse Serum

FBS: Fetal Bovine Serum

RPMI: Roswell Park Memorial Institute

DMEM: Dulbecco's Modified Eagle Medium

PBS: Phosphate Buffered Saline Solution

NaOH: Sodium Hydroxide

PMMA: Poly (Methyl Methacrylate)

IPA: Isopropyl Alcohol

PLL: Poly-L-Lysine

IDA: Interdigitated Array Electrode

CMP: Chemical-Mechanical Polishing

CVD: Chemical Vapor Deposition

PP: Polyprol

PCR: Polymerase Chain Reaction

SIN-1: 3-morpholinosydnonimine

GAP43: Growth Association Protein

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

EVALUATION OF NEURAL DIFFERENTIATION OF PC12 CELLS GROWN ON GRAPHENE COATED ITO MICROCHIPS

ABSTRACT

Nervous system diseases occupy a very large place among diseases without treatment today. One of the major challenges in nervous system diseases is achieving functional connection between nerve cells. A neuronal network is a very complex structure consisting of connections between neuronal cells through dendrites and axons. In this context, a human brain can be described as a complex neuron network. Platforms capable of creating a neuronal network have the potential to be used in a variety of fields, such as modeling neuron-related diseases, finding new treatments, building biological computers with the self-renewable capacity of living cells, as well as pharmacology. The design of different drugs requires testing in the same model. The biggest challenge in building a well-characterized neural network is the formation of desired bonds between cells and the absence of long enough and durable synapses. Synapse formation between cells provides communication and signals can be received from these cells to investigate some diseases and treatments. The first step of forming such connections is to induce spreading of neuronal extensions. In this study, the impact of graphene on neuronal differentiation of PC12 cells into neuron-like cells was evaluated in conjunction with electrical stimuli. First, an ITO microchip with a certain number of electrodes was fabricated using photolithography and then a chemically synthesized graphene was coated on the microchip. The electrical stimulation was applied through the ITO-microchip. Following optimization of neuronal differentiation conditions, the effect of AC and DC electrical stimulation on both bare and graphene-coated ITO-microchips for neuronal differentiation was investigated. According to the results, it was observed that electrical stimulation with direct current for 30 minutes caused a large degree of neuronal cell differentiation on the graphene coated ITO-microchips. The results were also verified by real-time qPCR. In the future, the surface of graphene coated microchips will be patterned with a polymer to form physical barriers that will guide the spreading of neuronal extensions in a particular direction.

Keywords: PC12 cells, ITO, microchip, neural differentiation, graphene

GRAFEN KAPLANMIŞ ITO MİKROÇİP ÜZERİNDE BÜYÜTÜLEN PC12 HÜCRELERİNİN NÖRONAL FARKLILAŞMASININ DEĞERLENDİRİLMESİ

ÖZET

Sinir sistemi hastalıkları günümüzde tedavisi olmayan hastalıklar içerisinde hayli büyük bir yer kaplamaktadır. Sinir sistemi hastalıklarındaki en büyük zorluklardan biri, sinir hücreleri arasında fonksiyonel bağlantılar kurabilmektir. Bir nöronal ağ, dendritler ve aksonlar vasıtasıyla nöronal hücreler arasındaki bağlantılardan oluşan çok karmaşık bir yapıdır. Bu bağlamda, bir insan beyni karmaşık bir nöron ağı olarak tanımlanabilir. Bir nöronal ağ oluşturma kapasitesine sahip platformlar, nöronla ilişkili hastalıkların modellenmesi, yeni tedavilerin bulunması, canlı hücrelerin kendi kendini yenileyebilen kapasitesine sahip biyolojik bilgisayarların inşası ve ayrıca farmakoloji gibi çeşitli alanlarda kullanım potansiyeline sahiptir. Farklı ilaçların tasarımı, aynı modelde test edilmesini gerektirir. İyi karakterize bir sinir ağının inşasında en büyük zorluk, hücreler arasında istenen bağların oluşturulması ve yeterince uzun ve dayanıklı sinaps oluşumunun olmamasıdır. Hücreler arasındaki sinaps oluşumu iletişim sağlar ve bazı hastalıkların ve tedavilerin araştırılması için bu hücrelerden sinyal alınabilir. Nöronal hücreler arasında bu tür bağlantıların oluşturulmasının ilk adımı nöronal uzantıların yayılmasını indüklemektir. Bu çalışmada, grafenin PC12 hücrelerinin nöron benzeri hücrelere nöronal farklılaşması üzerindeki etkisi elektriksel uyaranlarla birlikte incelenmiştir. İlk olarak, belirli sayıda elektrot içeren bir indiyum tin oksit (ITO) mikroçipi fotolitografi kullanılarak üretilmiş ve daha sonra mikroçip kimyasal olarak sentezlenmiş bir grafen ile kaplanmıştır. Nöronal farklılaşma koşullarının optimizasyonunun ardından, AC ve DC elektrik stimülasyonunun, hem çıplak hem de grafen kaplı ITO-mikroçipleri üzerinde nöronal farklılaşmaya etkisi araştırılmıştır. Sonuç olarak, 30 dakika boyunca doğru akım ile elektriksel stimülasyonun grafen kaplı ITO-mikroçiplerde büyük ölçüde nöronal hücre farklılaşmasına neden olduğu gözlemlenmiştir. Sonuçlar gerçek zamanlı qPCR ile de doğrulanmıştır. Gelecekte, grafen kaplı mikroçiplerin yüzeyi, nöronal uzantıların belirli bir yönde yayılmasına rehberlik edecek fiziksel bariyerler oluşturmak için bir polimer ile desenlenecektir.

Anahtar Kelimeler: PC12 Hücreleri, ITO, mikroçip, nöral farklılaşma, grafen

1. INTRODUCTION

1.1 Nervous System

Nervous system is one of human body systems that plays role in sending and receiving signal into brain or several body parts. Nervous system works as responding the changes from environment [1]. Nervous system in human body is divided by two categories and these are peripheral nervous system and central nervous system. Central nervous system is control center that include brain and spinal cord and the signals are transmitted into brain by neurons [2]. There are some different types of nerve cells in central nervous system. The autonomic nervous system (ANS) is classified into parasympathetic and sympathetic (SNS) also both the SNS and PNS composed of post-ganglionic and pre-ganglionic neurons on biological, physiological and pharmacological grounds. Pre-ganglionic SNS fibers come from the thoracolumbar areas of the spinal cord, with pre-ganglionic PNS fibers originating from craniosacral areas. In both the CNS and PNS, pre-ganglionic transmission is mediated through acetylcholine (ACh), acting on nicotinic acetylcholine receptors. Post-ganglionic signaling in SNS neurons is mainly regulated by noradrenaline, which works by specific adrenergic receptors, particularly in the sweat and adrenal glands. Post-ganglionic neurons in the sweat gland express ACh. The adrenal glands' pre-ganglionic fibers synapse immediately with the adrenal medulla, inducing adrenaline production from enterochromaffin cells. Adrenergic receptors are grouped into three main categories (β, α_1 and α_2), each form has more subgroups [3].

1.1.1 Nervous system mechanism

Neurons (nerve cells) consist of axons (long fibers of neurons) cell body part (soma) and dendrite. Cell body includes dendrite and nucleus. Also in some neurons, along the axon part of cell there is myelin sheath for fast transmission of signal [4]. Myelin sheath is formed when the Schwann cells wrapped. (See Figure 1.1) In nerve cells,

electrochemical signal transmitted along long fibers is called axon and at the end of axon release neurotransmitter to junctions are named synapses [5]. Chemical communication between nerve cells is done by hormones or neurotransmitters. Hormones are chemical message carriers between cells in the body via blood circulation and they are secreted from adrenal gland to the blood besides neurotransmitters secreted from axon terminal of nerve cell to another cell. There is also a term as “neurohormones” that secreted from nerve cells and spread by axon terminal to the blood not the post-synaptic cell [6].

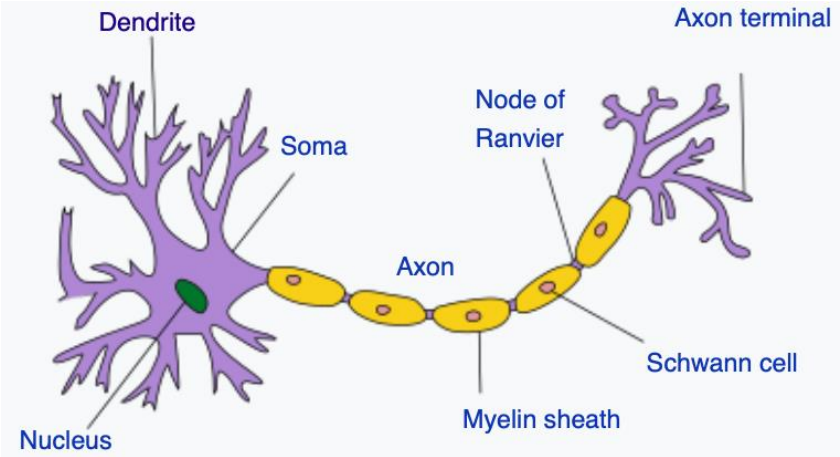

Figure 1.1 : Nerve cell and its parts [7].

1.1.2 Neural communication

There are four types of neural communication and they are primitive intercellular, neural, neurohormonal and hormonal communication. Primitive intercellular communication occurs between 3 or more cells for complex modes of communication. Neural communication occurs between neuron and target cells directly by releasing neurotransmitter from axon terminals of nerve cell. Neurohormonal communication occurs between nerve (neurosecretory) cells and target cells via blood circulation it means neurohormone released to blood and by blood circulation target cells are taken neurohormone inside itself. Hormonal communication occurs between endocrine and target cells via blood stream [6]. (See Figure 1.2)

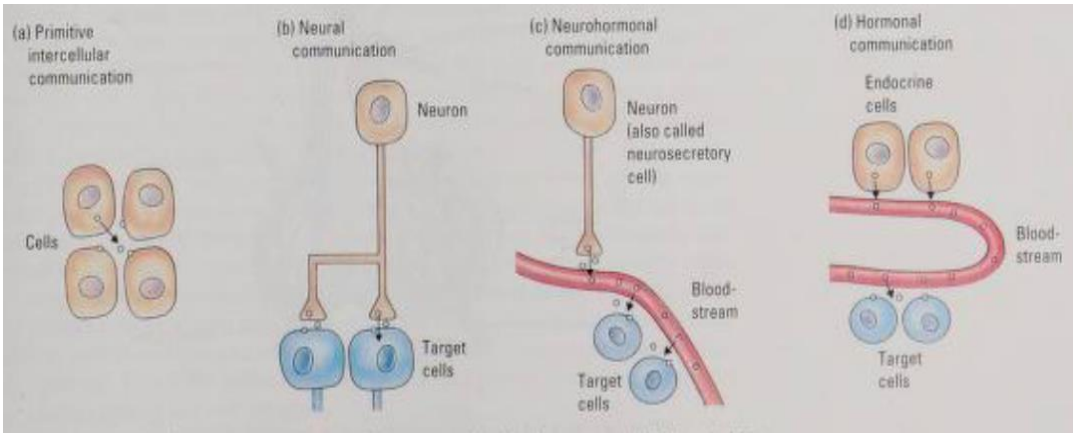

Figure 1.2 : Neural communication types [5].

1.1.3 Diseases and treatment about nervous system

Diseases related to nervous system lead to neurological disorders in brain and spinal cord. It can be caused by function deterioration of structure and mechanism of nerve cells. Neurological diseases show various side effects, yet might be brought about by a huge number of obscure reasons and factors. Neurological diseases are characterized into 3 groups that named neurodegenerative (Hungtinton's, Parkinson’s, Alzheimer’s diseases) neurotraumatic (stroke, spinal cord and brain injury) and neuropsychiatric (autism, depression, hyperactivity disorders) diseases. The significant fundamental functions leading to neurodegenerative disorders are multifactorial like hereditary, ecological factors as aging. [8].

Neurodegenerative diseases occur in the lately life. Older people have a high risk of being a neurodegenerative disease. Capillary electrophoresis (CE) is the isolation of solutes dependent on specific movement speeds via an electrical field over a substrate electrolyte where anions travel towards the anode and vice versa. The charge and size have an opposite influence on the motion of the charged particles [9]. Capillary Electrophoretic method is used in neuroscience applications also CE-laser-induced fluorescence is frequently utilized in neurological field studies, because of its high affectability [10].

Alzheimer’s disease lead to increase oxidative stress and neuroinflammation because of neurofibrils and amyloid plaques. Also, Parkinson’s disease is related to occur Lewy particles and neuronal death. Generally neurodegenerative diseases as Alzheimer’s and Parkinson’s tried to treat with drugs but because of blood brain barrier (BBB)

drugs cannot pass efficiently into brain. Blood brain barrier is natural barrier in brain between blood on circulation and central nervous system. BBB plays role in regulating ion and molecule balance so protect the neuron as preventing the harmful particles passing from brain fluid. BBB is also so selectively permeable and it prevents the drug of neurodegenerative diseases by blood circulation. Because of BBB some noninvasive and invasive therapeutic studies are performed in drug-delivery system. Drugs are directly entered into the brain as injecting these drugs to achieve the BBB efficiently in invasive therapeutic studies. In noninvasive therapeutic studies, drugs are carried with nanoparticles (NPs) that include peptides are cell-penetrating.

Nanotechnology applications in medicine have so many advantages beside able to cross BBB as biocompatibility, biodegradable, stable in blood circulation as a long time, more solubility, less toxicity, cost-effective, noninflammatory and easy delivery to specific area [11].

1.1.4 Nerve regeneration

Damage to human central nervous system (CNS) is one of the most challenging problems although there have been a lot of studies and treatment strategies related to that. While multiple repair approaches were shown to facilitate axon outgrowth in human CNS following neuronal injuries or damage, it still remains unknown if regenerated axons develop usable synapses and help behavior [12]. Generally, damages and injuries into CNS are permanent so it cannot be recovered or functions of nerve cell is highly limited. The reconstruction of normal nerve function requires a concerted reconstruction not only of the injured axons, but also of the myelin sheath, which coils around the axons of neurons facilitating saltatory nerve conduction and supplying trophic help. While peripheral nerves exhibit axonal regenerative properties, remyelination after nerve damage and disease in human brain happens at a slow speed and is sometimes impaired, resulting in deficits in the functional reinnervation of target tissues and recovery of sensorimotor activity [13]. Remyelination is the process of generating new myelin sheats that are found on axons in the CNS. The mechanism of remyelination relies through a well-characterized community of Oligodendroglial precursor cells (OPCs), capability of differentiating into mature oligodendrocytes that form myelin [14]. Remyelination technique can be used in loss of myelin sheat

diseases as Multiple Sclerosis (MS). OPCs can be integrated through lesions on ms nervous systems where they induce differentiation. Remyelination can recover behavioral functions to axons (hence restore neuronal function) and is generally thought to play a neuroprotective role on axons. Remyelination happens at few MS lesions but is becoming progressively insufficient and lacking in most lesions and patients [15].

1.1.5 Electrical stimulation effect on nerve regeneration

Electrical stimulation (ES) is one of the most challenging studies about nerve regeneration on both peripheral and central nerve injuries and damage for promoting axonal outgrowth. On the huge nerve defect, generally nerve graft is required as behaving a bridge between two nerve stumps. Nerve graft establish connections across which regenerating axons replicate to regain motor activity inside the distal nerve stump. tie ES as applied to the proximal nerve junction was shown to facilitate nerve regeneration. However, preceding studies have not documented the potential positive effect of creating a local electrical condition within a significant nerve failure on nerve regeneration. Setting up an electrical condition with ES situated at the conductive and biocompatible scaffold is capable of speeding nerve regeneration and promoting effective regeneration in rat brain tissue with a 1,5 cm nerve defect [16]. Another study showed that electrical stimulation (20 Hz, 100 mikrosaniye, 3 V) increased the quantity of remyelination as looking at gene (P0 and brain-derived neurotrophic factor (BDNF)) expression profiles and co-cultured cells and tissues. ES elevates the thickness of myelin sheat and promotes regeneration as axonal outgrowth [17]. Huang and coworkers showed also that in impaired peripheral nerve damage in vivo, electrical stimulation (ES; 3 V, 20 Hz, 20 min) accelerates the nerve regeneration and functional healing as providing the recovery in slow rate nerve regeneration. They observed that ES greatly increased the size and level of regenerated axons, the width of myelin sheath, and also the amount of retrograde-labeled Fluoro-Gold motoneurons and sensory nerve cells. indicating that brief ES to proximal nerve stumps is capable of fostering nerve regeneration in delayed nerve injury repair with differing prolonged periods of time, with the longest 24 weeks [18]. Gordon showed that low-frequency electrical stimulation (LFES) speeds up motor and sensory axon outgrowth through

the nerve injury points, promoting nerve regeneration and target reinnervation even after prolonged surgical reconstruction of damaged nerves in clinics [19].

1.2 Microchip

1.2.1 What is microchip?

Microchips are lab-on-a-chip (LOC) devices (Figure 1.3) that can manage and manipulate fluid flows at micro levels. Integrating these devices with biosensors provides their implementation in some application areas as pharmaceuticals, medical diagnostics, environmental monitoring and agriculture, and. A biosensor should own a biological response then converted to an electrical signal as magnetic, optical, electrochemical (EC), thermometric, piezoelectric and micromechanical terms. Fabrication of miniaturized equipment of microchip can withstand micro and nanotechnology techniques. It is comparable to the semiconductor revolution that has been radically impacted via lithographic processes. Integrating more than one laboratory features on a nano or micro-dimension provides obtain high-throughput automation and screening. Fabrication of microchip rely upon on various parameters, which includes pressure gradients, capillary forces and electrokinetics (EKs). For achieving excessive evaluation charge and reduced time and costs, microfluidic gadgets manage low-volume samples. It can be used for chemical analyses and synthesis [20].

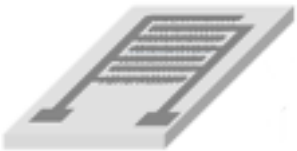

Figure 1.3 : Indium tin oxide glass microchip schematic diagram.

1.2.2 Advantages of microchips

Due to small volumes of analyte that is used in microchip, reaction time, reagent volume and costs decrease. These properties are important for serial production.

- Easy to use for analytical applications
- Quick answer for reducing reaction times
- High surface-to-volume-ratio
- Easy controlling of process

- Compact system
- Low cost for fabrication
- Could be disposable chips
- Provides safe application area as chemically

1.2.3 Use of microchip

Use of microchips is very large as clinical, research, industrial, environmental and others. First it has been used for research as analytical devices in 2014 and then drug discovery and development field. First time at clinical use is point of care then drug delivery and patient monitoring. In environmental and industrial use, it used as first agribusiness then toxicity monitoring and food testing respectively. Other fields are forensic science, stem cells and synthetic biology and many fields are coming in the future applications.

1.2.4 Materials for microchip

Materials for microchip were listed below (Table1.1).

Table 1.1 Materials for microchip.

Materials	**PDMS (Polydimethyl siloxane)**	**SU-8 (Epoxy Resins)**	**Silicon**	**Glass**	**Paper-based**
Properties	Necessary for surface modification High absorption capacity	High stability at high temperature Transparency Chemical resistance	Good solvent resistance Good thermal stability	Optically transparent Good insulator for electricity Resistive to chemicals High cost for fabrication High hardness	High porosity Easy to fabricate Low cost Easy to use High sensitivity High resolution

1.2.5 Fabrication Methods

1.2.5.1 Photolithography

Photolithography is a microchip fabrication method and also called UltraViolet (UV)-lithography that used in microelectromechanical systems (MEMS) device production. Photoresist is a light sensitive polymer that is used for this technique to obtain the shape of base shape by transferring the geometric shape on a mask to the surface of the silicon wafer (subsrate). In this method, depth lithography, soft lithography and injection molding are included [20].

1.2.5.2 Micromachining etching techniques

For the realization of miniaturized mechanical parts, this technique requires selective removal of the substrate content. Bulk micromachining may be done using different physical and chemical processes, with the use of chemical means in the MEMS indus try being much broader. The most used process of this technique is wet chemical ethcing of thin films and then dry etching technique is used. Chemical wet etching gives some advantages for MEMS as high selectivity and etch rates. In this method, wet chemical etching of thin films and dry etching techniques are included.

1.2.5.3 Bonding methods

Interacting 2 or more bio-components is intended to generate enclosed liquid tunnels or capilleries and cavities, and 3D composite materials, impart mechanical strength, produce thermal bonding, or provide electrical connection among others. Bond is formed at the wafer (subsrate) level, and the bonding materials depend on each technique. In this method, silicon direct bonding, anodic bonding, glass bonding, plasma-activated bonding, solid-liquid interduffusion bonding, adhesive bonding and glass frit bonding is included.

1.2.5.4 Maskless patterning techniques

Photolithography methods takes a very long time because of mask using but if mask isn’t used or digital methods are used, these provides speed of micromachining with a wide range of assisted materials and resolutions of micro-meter. In this method, laser micromachining, inkjet printing, 3D printing, micro electrical discharge machining, micromachining techniques are included.

Table 1.2 Fabrication methods of microchip.

Photolithography	Micromachining Etching Techniques	Bonding Methods	Maskless Patterning Techniques
Depth Lithography Soft Lithography Injection Molding	Wet Chemical Etching of thin films Dry Etching Techniques	Silicon Direct Bonding Anodic Bonding Glass Bonding Plasma-activated Bonding Solid-liquid interduffusion Bonding Adhesive Bonding Glass Frit Bonding	Laser Micromachining Inkjet Printing 3D Printing Microelectrical discharge Machining Micromachining Techniques

1.2.6 Detection mechanism of biosensors

Detection mechanism of biosensors are listed below. (Table 1.3)

1.2.6.1 Chemical

In chemical sensing, chemical sensors detect directly chemical substances related to their precise quantities. Mass spectrometry and luminescence is used for chemical sensing. While mass spectrometry detects the precise substance in the sample zone by the quantity difference, luminescence used light from markers in the sample.

Advantages:

- High mass accuracy
- High sensitivity
- Sufficient energy to excite an electron
- Naturally occurs
- Potential characterization tool

Disadvantages:

- Large sample volume

- Isomers of a molecules are non-distinguishable
- Low frequency of measurement
- Limited selectivity

1.2.6.2 Optical

In optical detection method generally fluorescence detection is used but the system is the same nearly. Molecules or biological sample (e.g. photon in fluorescence) is absorbed by sample and absorbing material gives re-emitting photon and wavelength between the absorbed and re-emitting photon is calculated by fluorescence techniques. In laser technique, light is emitted as the same processes.

Advantages:

- Fast
- Compact
- Simple
- Integrated process
- Real-time detection
- High sensitivity

Disadvantages:

- Dye emission light concealed by the excitation light
- Sensitive to surroundings
- Delicate process
- Bulky
- Need surface modifications

1.2.6.3 Electrochemical

In electrochemical detection, electrochemical sensors and detectors are used. In chemical sensors divided into 3 electrodes as reference electrode (RE), counter electrode (CE) and working electrode (WE). Working electrode plays role in sensing and electrical potential applied between counter and working electrode then counter electrode is measure the current by redox reactions on sample electrochemically.

Advantages:

- Economical
- Continuous analysis possible
- Simple
- Real-time detection

Disadvantages:

- High sensitivity
- Detection time takes long
- Current is related to redox species
- Bulky
- Sensitive against to temperature

1.2.6.4 Mechanical

In mechanical detection, the sample is measured by mechanically and cantilever and quartz crystal microbalance are sub-units of mechanical detection. Generally, in mechanical detection, mechanical quantities are used and measure the change of frequency based on the cantilever or quartz crystal microbalance.

Advantages:

- Compact
- Simple
- Real-time detection
- High sensitivity

Disadvantages:

- Sensitive to temperature and background
- Bulky

Table 1.3 Main detection mechanisms for microchip.

Detection Process	**Techniques**
Chemical	• Mass Spectrometry • Luminescence
Optical	• Fluorescence • SPR (Surface Plasmon Resonance)
Electrochemical	• Amperometric • Voltammetric • Impedimetric
Mechanical	• QCM (Quartz Crystal Microbalance) • Cantilever

1.2.7 Microchips for tissue engineering applications

Microchips have been used for several microbiological areas besides tissue engineering applications. Using microchip provides some advantages for analysis of cell culture systems since the size of fluid micro-gaps in the microchip is ideal for accommodating biological cells [21].

Some physical properties are researched on the microchips such as shear stress related to changes of their morphology [22, 23]. Also using neuronal cells, their secreted molecules are analyzed and detected by microchips [21, 24]. In one research, non-fluorescent molecules are detected by using photothermal process via fluorescent probes [25]. Generally sub-cellular molecules are non-detectable so for detecting these molecules, fluorescent markers are used with high sensitivity and selectivity. Due to small gaps and ease to liquid control of microchip allows to detect these molecules and there are scanned with thermal lens microscope.

Microchips are used in bioassay systems. In this system, organic cells such as myoblasts, endothelial cells and hepatocytes are cultured onto/into microchip for bioassay systems [26-30]. Controlling of bacterial populations and growth of yeast colony studies can be done with microchips [31, 32]. There are many techniques for detection of micro bioassay systems as explained previous section.

Microsystems have been used for mimicking some organs and tissues. In tissue engineering application, microchips are widely used for developing mimic organs or medical devices for body [33-36].

1.3 Graphene

Graphene is a form of carbon that is called allotrope. Basically, carbon nanotubes, graphite and fullerenes are also allotropes of carbon element. When graphene is compared to other allotropes, graphene has good properties as thickness, being strong and lower density [37, 38]. Besides these properties, electrical conduction of graphene is extremely high [39-46]. Graphene is mostly used for decades in many application areas (biomedical, composites and coatings. Electronics, energy, membranes and sensors) due to these good properties.

1.3.1 Properties of graphene

Graphene has specific properties according to other allotropes of carbon element and these properties are listed below (Table 1.4) [39-46].

Table 1.4 Properties of graphene, their subsections and mathematical values.

Mechanical Properties	Tensile strength	130 GPa
	Young's modulus	1 TPa
	Tension rigidity	340 GPa·nm
	Surface tension	54.8 mN/m[8]
	Flexural rigidity	3.18 GPa·nm^3
	Thermal conductivity	2-4 kW·m^{-1}K^{-1}
	Distance between adjacent layers of graphene in graphite	3.4 Å
Chemical Properties	Burn temperature	350 °C (662 °F)
	Specific surface area	1168 m^2·g^{-1}
Electrical Properties	Band gap (in sheet graphene)	0 eV
	Electron mobility (intrinsic limit)	-200,000 cm^2·V^{-1}·s^{-1}
	Electron mobility (on SiO_2 substrate)	~40,000 cm^2·V^{-1}·s^{-1}
	Carrier density	10^{12} cm^{-2}
	Free path for electron-acoustic phonon scattering	>2 µm
Optical Properties	White light absorption:	2.3 %
	White light transmission	97.7 %

Graphene has different forms as monolayer sheets, superlattices, bilayer graphene, graphene oxide, nanoribbons, 3D graphene, quantum dots, graphene fiber, graphene ligand, molded graphene, pillared graphene, nanocoil and graphene aerogel.

1.3.2 Graphene production techniques

Graphene is used for many research applications so graphene production techniques are extremely wide. Due to its properties, it has different types of production techniques [47]. Various techniques have been successfully adopted to produce graphene (Table 1.5).

Table 1.5 Graphene production techniques and their subsections.

Production Techniques	1.Exfoliation	1.1.Adhesive tape
		1.2.Wedge-based
		1.3.Graphite oxide reduction
		1.4.Shearing
		1.5.Sonication
		1.6.Molten salts
		1.7.Electrochemical synthesis
	2.Epitaxy	2.1.Chemical Vapor deposition
	3.Epitaxy of graphene	3.1.Silicon carbide
		3.2.Silicon/germanium/hydrogen/
		3.3.Metal single crystal substrates
		3.4.Ruthenium
		3.5.Iridium
		3.6.Platinum
		3.7.Nickel
		3.8.Cobalt
		3.9.Copper
		3.10.Tin
		3.11.Sodium ethoxide pyrolysis
		3.12.Roll-to-roll
		3.13.Cold Wall
	4.Nanotube slicing	
	5.Langmuir-blodgett (LB)	
	6.Carbon dioxide reduction	
	7.Spin coating	
	8.Supersonic spray	
	9.Intercalation	
	10.Laser	
	11.Microwave-assisted oxidation	
	12.Ion implantation	
	13.Heated vegetable oil	
	14.Bacteria processing of graphene oxide	
	15.Flash joule heating	
	16.Hydrothermal self-assembly	

1.3.3 Graphene applications

Graphene is mostly used in production of low cost and high productivity photovoltaic devices, smart phones and battery-based touch panels application due to its high transparency and conductivity [48, 49].

1.3.4 Graphene microchips

Biosensors provide to detect and analyze the components from the surface of electrode with high sensitivity, detectability, selectivity and biocompatible features. For the evaluation of physiological and metabolic parameters, biosensors with high sensitivity, selectivity and a low detection limit, approaching nano / picomolar concentrations of biomolecules are essential to the medical sciences and healthcare industry. Many biological components have been detected for biosensors including cancer biomarkers, antibodies, hydrogen peroxide, glucose, bacteria, heavy metals and any other biomolecules [50-62]. Several nanomaterials have been used during the last decade to develop extremely effective biosensors for the identification of biomolecules from analytes. Due to its unique physical properties including high carrier mobility, high specific surface area, high adaptability, conductivity, and optical transparency, the discovery of graphene has amazingly highly effective into the manufacture of low-cost electrodes [63, 64]. Graphene and its derivatives are graphene oxide (GO) and reduced graphene oxide (rGO) nanosheets can be conveniently mixed with different forms of nanoparticles, including semi-conductive nanoparticles, metals, quantum dots, metal oxides, inorganic and organic polymers, and biomolecules, to construct a wide variety of graphene-based nanocomposites with improved sensitivity for biosensor applications [65-67].

Combining graphene and ITO improved the photovoltaic characteristic and reduced charge loaded recombination relay [68]. Graphene coated ITO with nanocomposites are used for electrode to detect some biomolecules. Xinrui et al. sowed that the MoS_2 nanoflowers (NFs)-reduced GO/ITO working electrode provides to detect uric acid and dopamine due to large holes in the working electrode so bioactive substance could enter rapidly and well. This hybrid working electrode had a high sensitivity 2.29 and 1.88 $\mu A\ \mu M^{-1}$ low detection limit and it also shows good stability so provides to detect real biological substances [69]. Graphene used for detection of some biomolecules that are important for human body in health care field. In one study, poly (pyronin Y) modified with graphene onto ITO glass and then it used for again as an electrode in electrochemical cell so according to results this graphene nanocomposite ITO film can be used as electrode for electrochemically detection of nitrite [70]. In another study, graphene coated ITO film used as electrode for enhance the electrooxidation facility

of glucose molecule and the best chemically active non-oxygenated ring C atom of graphene is chosen then G7 modified ITO electrode showed better specificity according to other electrodes in literature [71]. In one study gold/graphene nanostructure used for modification on ITO film to make an electrode for electrochemically detection of Aflatoxin B1 and results showed that low detection limit as 6.9 pg/mL [72].

1.4 PC12 Cell Line

PC12 cell line is a single cell clonal line that was obtained from transplantable rat adrenal pheochromocytoma. For researchers that are studying on nervous system, clonal cell line is a perfect model at molecularly in single cell [73]. A study showed that transplantable rat pheochromocytoma had a potential to differentiate and cell line derived from tumor tissue then cultured for neural differentiation in the presence of nerve growth factor (NGF) [74]. The cells are semi-suspended cells so culture flasks needed to coating with Collagen IV or Poly-d-lysine [75]. Medium of culturing cells is needed to refresh every 2 or 3 days. Freeze medium of cells is complete growth medium with 10% (v/v) Dimethyl sulfoxide (DMSO). Cells must be kept in the liquid nitrogen vapor phase in liquid nitrogen tanks for storage. There are different complete medium ratios of donor horse serum (DHS), fetal bovine serum (FBS) and different antibiotics in RPMI or DMEM mediums. Cultured cells must be in the cell culture incubator with 5% CO_2 at 37°C. Doubling time of cells is 48 hours [74].

PC12 cell line is used in research are of medicine and combined with many field as nanotechnology, biomechanic, tissue engineering, biomaterials, genetic, biochemistry biosensor and etc. [76-81]. Released biomolecules from PC12 cells are detectable so there are many studies about detecting some substance from PC12 cells in biosensor field. Chen et al. designed an electrochemical biosensor and detect the nicotine-induced dopamine secreted from PC12 cells with high sensitivity of 17.0134 μA cm2 μM^{-1} and detection limit of 1.2 nmol L^{-1} (S/N = 3) [82]. Matsuba et al. produced a calcium and potassium ion image sensor and then observed the acetylcholine induced PC12 cells are biologically imaged. In sensor field calcium and potassium is really important for signaling so this study provides to analyze the cell metabolism by difference of calcium and potassium ion concentrations [83]. Zheng et

al. fabricated a nanoelectrodes then modified with graphene at the end of the fabrication electrode was functionalized with polyaniline. Surface of electrode had wide holes for electron transfer so observed that the nerve regeneration of PC12 cells by applying electrical stimulation. The results showed that this study can be the good strategy of neuronal diseases and traumatology by improving nerve regeneration with enhanced neurite outgrowth [84].

1.5 The Aim of the Study

The primary goal of the study was to evaluate the impact of graphene in conjunction with electrical stimulation on neuronal differentiation of PC12 cells into neuron-like cells. To that end, an ITO microchip with a certain number of electrodes was fabricated using photolithography and then a chemically synthesized graphene was coated on the microchip. Following optimization of neuronal differentiation conditions, the impact of AC and DC electrical stimulation on neuronal cell differentiation was investigated, respectively on both bare and graphene coated ITO-microchips. Lastly, a real-time qPCR test was carried out to verify the obtained results.

2. MATERIAL & METHOD

2.1 Materials

Collagen (type IV, 0.5-2 mg/ml) (Sigma-Aldrich, USA), Acetic Acid (Sigma-Aldrich, USA), Poly-L-Lysine (Sigma-Aldrich, USA), Trypsin (Gibco, USA), donor horse serum (DHS) (Capricorn, Germany), fetal bovine serum (FBS) (Sigma-Aldrich, USA), L-Glutamine (Gibco, USA), Gentamycin (Gibco, USA), RPMI 1640 (Sigma-Aldrich, USA), Nerve Growth Factor (NGF) (Vipera lebetina venom) (Sigma-Aldrich, USA), Phosphate buffered saline solution (PBS) (Sigma Aldrich, USA), NaOH (Sigma-Aldrich, USA), Irgacure (Sigma-Aldrich, USA), copper (Sigma-Aldrich, USA), hydrogen gas (H2) (Sigma-Aldrich, USA), argon gas (Ar) (Sigma-Aldrich, USA), poly (methyl methacrylate) (PMMA) (Sigma-Aldrich, USA), acetone (Sigma-Aldrich, USA), IPA (isopropyl alcohol) (Sigma-Aldrich, USA), $FeCl_3$ (Sigma-Aldrich, USA) and ethanol (Sigma-Aldrich, USA), AZ5214E reversal photoresist (MicroChemica ls, Germany), AZ1505 (MicroChemicals, Germany), AZ400K developer (MicroChemicals, Germany), AZ 100 develover remover (MicroChemicals, Germany), Calcein AM Kit (Biotium, USA), MTT Cell Proliferation Assay Kit (ThermoFisher, USA), DMSO (Sigma-Aldrich, USA), live/dead double staining kit (Dojindo, Japan).

2.2 PC12 Cell Culture

2.2.1 Coating

Since the cells are semi-suspended cells due to their nature, surface coating was needed. All coating processes were completed in the cell culture cabinet to avoid any contamination and just in case the coated flasks were UV sterilized before use. The surface was first coated with poly-L-Lysine (PLL), some of the cells were grown on this coating, and some were grown on collagen-coated surfaces. Commercially purchased PLL for coating was first used, diluted 10 times and 20 times with PBS, without diluting in three groups. To make coating, 2.5 ml solution from each group was added to three different 25 cm^2 flask surfaces and left in an incubator for 5 minutes. Excess solutions in flasks from the incubator were drawn with a pipette. The

flasks were then dried at room temperature and stored at +4 ° C for cell growth. For the collagen coating, 5 mL of PBS solution containing 0.25% acetic acid was added into 0.5 mg of collagen commercially purchased. At 2-8°C, the solution was shaken until homogenized. After the solution was homogenized, it was transferred to small ependorph tubes and removed for storage at -20°C in order not to spoil the protein structure of the collagen by subjecting it to freeze and thaw again. The coating protocol, on the other hand, was dropped onto the flask surface with the help of a pipette until it is sure that it covers the surface and then the solution is pipetted and released. The flasks were then incubated in the incubator for 2 hours to coat the surface with collagen. The flasks, which were observed to be completely dry after incubation, were stored at +4°C for later use. Cells were also passage into flasks coated with both solutions, and according to the results, it was observed that collagen was more easily attached to and grown faster due to its protein structure, so experiments were continued with collagen coated flasks.

2.2.2 Thawing

Pre-frozen cells were thawed in the water bath until half dissolved then centrifuged in a "falcon" tube (centrifuge tube) at 1000 rpm at +4 ° C after 1 ml RPMI 1640 (10% DHS + 1% FBS + 1% L-Glutamine + 0.1% Gentamycin) homogenized with the help of vortex in the medium. This cell suspension is then transferred to cell culture flasks that is coated before with containing 4 ml of RPMI 1640 (10% DHS + 1% FBS + 1% L-Glutamine + 0.1% Gentamycin) medium. Subsequently, these flasks are placed in an incubator containing 5% CO_2 at 37°C, allowing the cells to grow in these flasks.

2.2.3 Culturing

The purchased cells are centrifuged in a "falcon" tube (centrifuge tube) at 1000 rpm at +4 ° C after 1 ml RPMI 1640 (10% DHS + 1% FBS + 1% L-Glutamine + 0.1% Gentamycin) homogenized with the help of vortex in the medium. This cell suspension is then transferred to cell culture flasks that is coated before with containing 4 ml of RPMI 1640 (10% DHS + 1% FBS + 1% L-Glutamine + 0.1% Gentamycin) medium. Subsequently, these flasks are placed in an incubator containing 5% CO_2 at 37°C, allowing the cells to grow in these flasks.

Firstly, the flask containing the cells was examined under a microscope and its density was observed, and the flask, which is between 80% and 90% cell density, was taken into the cell cabinet after disinfection with 70% ethyl alcohol. Firstly, the cells were washed with 5 mL PBS solution for approximately 30 seconds in order to remove the cells from the surface easily. After washing, 1 mL of trypsin solution was dropped and the flask containing the cells was taken into the incubator. After incubating in the incubator for about 5 minutes, it was checked whether the cells were removed from the surface under a microscope. After observing the lifted cells, the flask was taken back to the cell cabinet. 4 ml RPMI 1640 (10% DHS + 1% FBS + 1% L-Glutamine + 0.1% Gentamycin) medium was added to the flask containing trypsin solution. With the help of a pipette, the cell in the flask was transferred to the suspension centrifuge tube (falcon) with a trypsin and RPMI 1640 (10% DHS + 1% FBS + 1% L-Glutamine + 0.1% Gentamycin) medium. The falcon was centrifuged at 1000 rpm + 4°C for 5 minutes to precipitate the cells and obtain clean. After the centrifugation process, the supernatant solution in the liquid form at the top was taken with the help of a pipette in order to use the collapsed cells at the bottom of the falcon. 5 ml RPMI 1640 (10% DHS + 1% FBS + 1% L-Glutamine + 0.1% Gentamycin) medium was added to the cells and the cells were suspended and 1 ml of the suspended solution was transferred to the collagen coated flask. It was passaged at the rate of 5:1. All stages of cell culturing were carried out in a clean push stove, in case of possible contamination, except the centrifuge and incubation sections.

2.2.4 Freezing

During the passaging after centrifuge, cells had been passaged and the remain homogenized solution were centrifuged again at 1000 rpm + 4°C for 5 minutes to precipitate the cells. The liquid on the cells were taken to trash and the freezing medium (10% DMSO and %90 FBS) were added on the remain suspended cells. Solution were mixed until it becomes homogenized by pipetting. The solution was transferred into the freezing vials that is resistant to liquid nitrogen tank. After that, the vial was placed to -80°C for placing to the liquid nitrogen tank after 2 weeks.

2.2.5 Viability with MTT assay

At this stage, the viability of the cells on the collagen-coated surface is under control over time. Cell viability is used for testing MTT (Vybrant ® MTT Cell Proliferation Test Kit) on days 0, 1, 4 and 7 3- (4,5-dimethylazol-2-yl) -2,5-diphenoltetrazole bromide) MTT, phosphate buffered salt in the solution, filtered through a 0.22 mm filter. In the well of the 24-well plate, 50 µL MTT solution was added to the well in 450 mL serum free medium. Plates were incubated at 37 ° C for 2 hours and then the medium was removed from the wells. 500 µL DMSO solution was added to each well in a light-free environment and then optical density measurements were made at 570 nm. (Synergy ™ HTX- BioTek, Winooski, VT, USA).

2.2.6 Cells staining

A "live / dead couple staining kit" was used to stain the cells for fluorescence imaging. The protocol included in the kit was applied for the staining procedure. Briefly, cells were washed 3 times with PBS solution. Then the washed cells were added 1 mL of calcein-AM / EthD-1 solution containing 2 µL of calcein-AM and 4 µL of EthD-1 (ethidiumhomodimer-1) and diluted with PBS in a ratio of 1: 100. Cells were incubated at 37°C for at least 30 minutes. The stained cells were then washed three times with PBS and images were obtained using a fluorescent microscope (Axio Vert.A1, Zeiss, Germany). Calcein AM is a dye that can pass through the cell membrane in most eukaryotic cells and can be used to determine cell viability. In the living cells, non-fluorescent calcein AM is converted into a green fluorescent calcein after acetoxymethyl ester hydrolysis with intracellular esterases.

2.2.7 Effect of nerve growth factor (NGF)

The most suitable differentiation conditions were determined for PC12 cells used by trying different NFG density in different medium combinations. First of all, NGF purchased for use later in the study is stocked. Phosphate buffer solution (PBS) containing 1 mg/mL protein was added to the NGF bottle in a sterile environment and the entire NGF contained in the bottle was dissolved in solution with the help of a vortex. Since it is known that freezing and thawing will decrease the effectiveness of the enzyme, NGF solution is divided into small ependorph tubes with a concentration of 500 ng/mL, each being 25 µL, and stored at -20°C. Cells that were removed and

centrifuged by trypsin during passage were added to a 96-well plate previously coated with collagen, with $1x10^3$ cells per well. Cells in 37°C incubator containing 5% CO_2 (EC160, core, Turkey) were placed were incubated for 24 hours. In the next step, each experiment group with 3 replicates was combined with two different media (serum medium and serum-free medium) and 3 different nerve growth factor (NGF) concentrations (0,100,200 ng/mL) and the cells were incubated for 6 days. Content of serum medium RPMI 1640 + 10% DHS (Donor Horse Serum) + 1% FBS (Fetal Bovine Serum) + 1% L-glutamine + 0.1% gentamicin content, content of serum-free medium RPMI 1640 + 1% DHS (Donor Horse Serum) + 1% L-glutamine + 0.1% gentamicin. The media exchange was repeated every 2 days for a healthy differentiation of the cells. The cells were examined and photographed under the microscope (CKX41, Olympus, Japan) every day to observe the results of the experiment, to see the effect of NGF and what medium to use.

2.3 Fabrication of ITO Microchip

In this part, the fabrication of the microchip and the production of masks for photolithography, which will form the basis for the new platform developed. Vectorworks program was used for microchip design. The distance between the electrodes was determined as 50-100 μm in order to achieve a successful result. It was thought that this distance will be sufficient for the extension of axon and dendritic structures. The design, which was output from the Vectorworks program in DXF format, was then converted to GDS format with the Layout program for the laser mask writing machine. 12.6 × 12.6 mm ready-made chrome mask was used in mask production. Chromium side was coated with AZ1505 positive photoresist using “spin coater”. Later, the prepared designs were transferred onto the mask with Heidelberg brand (DWL 66FS) laser mask writing machine. In the next part, the areas that become sensitive as a result of laser application were removed with a solution of “AZ400K developer” mixed with pure water in a ratio of 1:4, and the chromium located in the areas where the photoresist was removed was etched with sodium nitrate/perchloric acid solution, also known as chrome “etching” solution. The photoresist that remained in unetched regions where was removed with “AZ 100 developer remover”.

After the production of chrome masks, the fabrication of Indium Tin Oxide (ITO) interdigitated array electrode (IDA) microchips was made with use of photolithographic methods. Firstly, ITO coated glass substrates (slide) were cleaned with various liquids (ultrapure water, acetone, isopropanol) with ultrasonic methods and treated with oxygen plasma to remove undesired residues. Then the surface of ITO coated glass slides was coated with a positive/reversal photoresist AZ5214E via "Spin-Coater" at 2000 rpm for 60 sec. The polymerization of photoresist (pre-baking) was provided by keeping the photoresist-coated substrates in an oven set at 90°C for 30 minutes. In the following micro-production step, patterning was carried out using chrome lithography mask produced specifically for the microchip. Before this process, the lithography mask to be used were first cleaned with the help of pure water and acetone, and dried with a special dust-repellent cloth and nitrogen gun. The cleaned and controlled lithography mask was placed in the UV mask aligner. The photoresist coated slide to be used was removed from the oven and cooled for a while, then fixed under the mask with the help of double-sided tape. The shapes on the mask were exposed to UV light for 16 seconds with the help of UV mask aligner and transferred onto photoresist coated slides. In order to remove the UV-exposed areas, a cheap and effective solvent (developer) was prepared using a mixture of 0.8 g NaOH and 100 mL distilled water. The slides was separated from the mask and placed in the developer, which was prepared in order to remove the areas that become sensitive after UV light exposure (positive photoresist basic understanding) by shaking gently for 7 sec, so the necessary shapes for the microchip were passed on ITO coated slides with the photoresist. Afterwards, slides were rinsed in distilled water and dried with the help of nitrogen gun. In the last step, the control of the dried slides was carried out under a microscope. The checked microchips that were taken to the ion beam etching device for etching. Firstly, the device was operated with "Rough Pump", when the pressure dropped to the mtorr level, and then, the pressure dropped to the μtorr level with the turbo molecular pump. After the vacuum has been activated, the necessary parameters for etching have been entered into the device. For ITO-coated slides, etching was performed by rotating the sample holder at an angle of 22.5 degrees for 1 hour using parameters of 750 V, 49 W, 0.05 A with 30 sccm Argon gas. Since the photoresist coated part is resistant to this process, the parts where the photoresist was removed

were abraded on ITO-coated slides. After the microchips produced, they were checked under a microscope and with a multimeter to prevent possible short circuits. Finally, the photoresists on the remaining shapes were removed with acetone (See Figure 2.1).

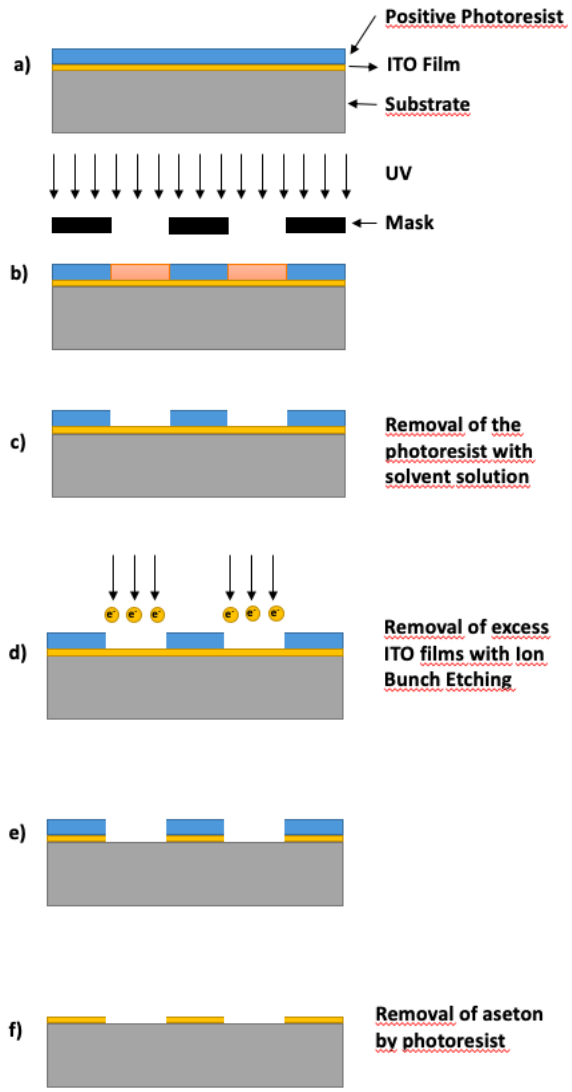

Figure 2.1 Schematic diagram of ITO Microchip fabrication

2.4 Electrical Stimulation of Cells on ITO Microchip

ITO microchips, which are washed with soapy water first and then with deionized water, are coated with collagen. Microchips that were sterilized overnight under UV light (260 nm) in the clean cabin were placed in petri dishes used for culture and made ready for cell cultivation. During the culturing of the cells, cells were counted with

$2x10^3$ cells per cm^2 and the cell suspension containing the medium was sown on the microchip. It was kept in a 37°C incubator containing 5% CO_2 for 2 hours to hold onto the microchip for 2 hours. At the end of the two hours, 5 mL of medium was added into the 60 mm petri dish where the microchip was placed. Adding neural differentiation factor to the medium and electrical stimulation processes were started 48 hours after the cells were planted on the microchip to ensure that the cells hold and spread to the desired level on the microchip. Cell behaviors with different parameters were observed using three microchips; The first microchip was given electric current at 100 Hz every day for 2 hours at 100 mV_{pp}/mm, the second microchip was applied with the same parameters throughout the day (24 hours) and the third microchip was used as the control group. (Figure 2.2) After electrical stimulation application, the medium of all samples was replaced with medium containing NGF. In addition, the cell medium was renewed every two days with medium containing 100 ng/mL NGF (Nerve Growth Factor). Cells were examined and images were taken daily under a microscope (CKX41, Olympus, Japan).

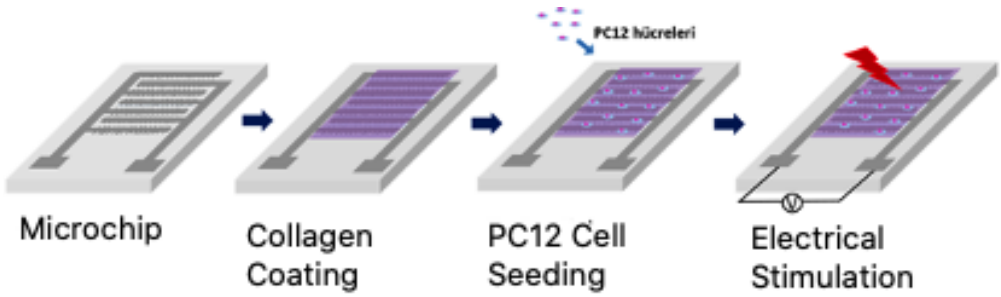

Figure 2.2 Schematic diagram of experiment procedure.

2.5 Graphene Synthesis and Coating

First, to reduce the density of impurities or defects on commercially available copper (Cu) foils, the foils were annealed at high temperature and the surface was cleaned by the chemical-mechanical polishing (CMP) method. Graphene was synthesized on polished copper and cleaned using chemical vapor deposition method at atmospheric pressure. Chemical vapor deposition (CVD) is the process of accumulating films in the vapor phase of materials by separating chemicals on the surface of a substrate. The Cu Foil was placed in a CVD quartz tube with hydrogen (H_2, 80 sccm) and argon (Ar,

1000 sccm) flow. The temperature was raised from room temperature to 1079°C and annealed with the same gas atmosphere for 30 minutes. Graphene was synthesized at 1079°C with a flow of carbon source methane gas for 1 minute, and then the medium in which it was found was cooled to room temperature in an Ar/H_2 atmosphere. After synthesis, poly (methyl methacrylate) (PMMA) was coated on graphene films and then left in acid solution ($FeCl_3$) for 1 hour to remove copper. After copper was completely abraded, graphene films containing PMMA were rinsed and cleaned several times with pure water. After the films were cleaned, they were transferred to the previously prepared ITO microchips with the dipping technique (fishing method). Graphene transferred ITO microchips were dried in the drying oven for 20 minutes. Finally, the protective layer, the polymer, was cleaned for 3 minutes in hot acetone, IPA (isopropyl alcohol) and ethanol, respectively, and dried with a nitrogen gun. Thus, the synthesized graphene was coated on ITO microchips (Figure 2.3).

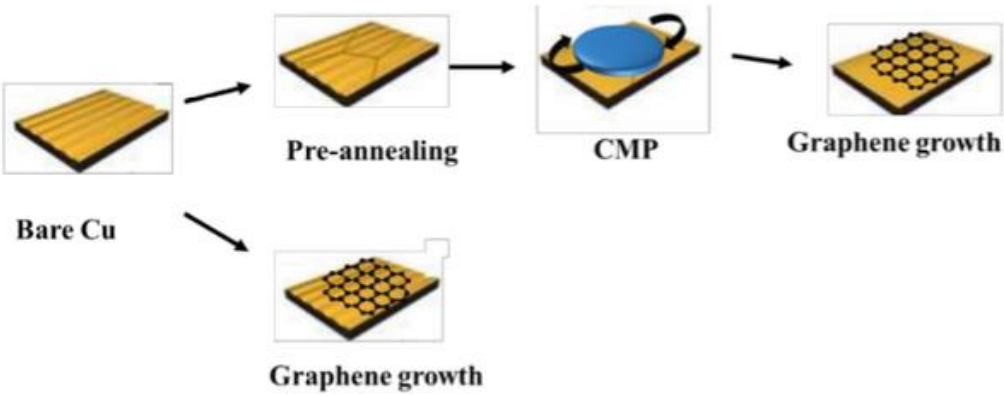

Figure 2.3 Schematic representation of graphene synthesis.

2.6 Electrical Stimulation of Cells on Graphene Coated-ITO Microchip

Firstly, graphene coated ITO microchips were sterilized overnight with UV light of the 2nd class safety biological cabinet to prevent any risk of contamination. The next day, samples taken from UV light were processed for collagen coating. Collagen solution (approximately 100 µl) in the amount to be collagen coated was dropped on each area of the graphene coated part and taken into the incubator (37.5°C) used for

cell culture containing 5% CO_2. Microchips that were left in the incubator until dry (about 2 hours) were taken back to the cell culture cabinet for sterilization. Microchips were sterilized under UV light for at least 30 minutes to minimize any risk of contamination in the cell culture cabinet. After the cell seeding process was done on the microchip, the microchip was taken into a 35 mm petri dish, since the cells would need a container to feed the required nutrient. Since electrical stimulation will be done after the seeding process, the wires of the microchip in the petri are fixed with the help of tape on the outer edge of the petri dish in order not to damage the cells. At the last stage of the passages, 5 ml of medium was removed and the cells were suspended and 1 ml of the suspended solution was transferred to collagen coated flask and passaged in a 5:1 ratio. As the remaining cell suspension will be sown on the microchip, counting is performed on the hemocytometer first, and then, after making the necessary calculations, $5x10^3$ cells are added to the cm^2 on the microchip. As PC12 cells are semi-suspended cells and not easy to hold, they were kept in the incubator so that they could hold onto the surface for half an hour. After the incubation, 5 ml of medium was added to the petri dish to grow the cells on the microchip and the cells were incubated for 24 hours until stimulation or continue to the experiments.

Graphene-coated ITO microchips with PC12 cells were incubated for 24 hours to allow the cells to adequately hold and reproduce, and then the necessary mechanism for electrical stimulation was established. Cable connections to be connected to the fixed wires were first checked with a multimeter. In the cover part of the incubator, the cables are first disinfected with 70% ethyl alcohol solution, then fixed with the help of tape and tied to the wires of the microchip in the petri dish.

Studies in the literature have been used to determine the parameters of the electrical stimulation to be applied. Chen and his team examined the effects of different levels of electrical stimulation on PC12 differentiation, and observed that in these conditions, 100 mVpp / mm (100 Hz) AC electrical stimulation had the most pronounced effect on neurite growth and the percentage of neurite cells in the presence of 100 ng / ml NGF [85]. In one study, Langer and his team reported that PC12 cells grown on oxidized polyprol (PP), which is an electrically conductive polymer, exposed to electrical stimulation of 100 mV DC showed a significant increase in neurite lengths compared to those not exposed to electrical stimulation [86]. 100 mVpp in the

induction of neural differentiation in the literature there are many studies using electrical stimulation at / mm [87, 88]. Therefore, in this project, 100 mV / mm electrical field was used for both AC and DC electrical stimulation of neural differentiation of PC12 cells.

Electrical stimulation was applied from the DC current wave source for 30 minutes to the first group, with a voltage of 100 mV_{pp}/mm and 100 Hz frequency parameretes to the first group to be examined, and 100 mV_{pp}/mm to the second group. Electrical stimulation was not applied to the third group of graphene coated ITO microchips to be used as a control group. After electrical stimulation, the cells were kept for a while (approximately 1-2 hours), although they were suspended, and then observed under a microscope. These cells were then taken into the cabinet and the medium was replaced with a medium containing 100 ng/ml NGF (Nerve Growth Factor). In order to observe the neurite extraction and elongation of the cells, the cells were taken back to the incubator and microscope images were taken on certain days.

2.7 Real-Time Polymerase Chain Reaction (RT-PCR)

For PCR, the mRNAs of the cells must first be extracted and translated into cDNA. A commercial kit was used for mRNA extraction. Firstly, the cells are washed normally with PBS solution 1-2 times and trypsin is added on the cells for lifting-off. For activation of trypsin, cells were placed in the incubator for 5 minutes. After waiting, cells were collected by adding PBS on the trypsin cell solution and suspended with a pipette and transferred to the ependorph tube. It was centrifuged at 13000 rpm for 5 minutes at room temperature. After centrifugation, the supernatant was removed by pipette. After adding 200 µl of RC buffer to the pellet and suspending with a pipette, it was homogenized with the help of vortex and waited 5 minutes to complete the chemical reaction. Then 300 µl RL buffer was added to the solution. Add 5 µl of 2-mercapto-ethanol and mix with vortex and waited 5 minutes. Then ependorph tube centrifuged at 13000 rpm in room temperature for 2 minutes. After centrifugation, 400 µl of new ependorph was placed in the supernatant part and sterile 400 µl ethanol was added on top and the entire solution was mixed with vortex. After placing the column with the collection tube in the RNA isolation kit, 800 µl solution was transferred into the tube column. After centrifugation at 13000 rpm for 1 minute at room temperature,

the tube was drained and the column was put back into the tube. A solution of 500 µl W1 solution was added to the top and the centrifuge was centrifuged at 13000 rpm for 1 min at room temperature, and then the tube was separated from the tube and the liquid was poured. The process was repeated by adding 600 µl W2 solution. W2 solution was added again and the process was repeated once more. In the same way, after separating the column with the tube and pouring the liquid from the tube, it was placed in the tube and centrifuged for 3 minutes at 13000 rpm without adding anything inside. After centrifugation, the column and tube were separated from each other and the column was placed in a new ependorph tube. There is a filter at the bottom of the column, 30 µl RNA Prewater solution was added in the middle of the filter and it was waited for 3 minutes. Then 3 minutes centrifuge at 13000 rpm. As a result of this process, mRNA molecules were collected in the ependorph tube.

Before converting the isolated mRNA molecules to cDNA, absorbance measurement was made in the NanoDrop device and the dilution rate was determined. According to the absorbance values, the best result was obtained in the dilution ratio of 1:10. Samples were taken to convert mRNA molecules into cDNA. During this process, the QuantaBio cDNA synthesis kit was used and all procedures were performed according to the protocol included in the kit. Briefly, 10 µl pure water, 4 µl mix and 1 µl RT solution were added to 5 µl mRNA solution. After mixing with vortex, centrifugation was performed for 10 seconds. The solution placed in the PCR device was subjected to different temperatures at varying times for the conversion to cDNA; 5 minutes at 22°C, 30 minutes at 42°C, 5 minutes at 85°C, and finally the temperature was lowered at 4°C. Thanks to these processes, mRNA molecules have been converted to cDNA.

For PCR processing, cDNAs were diluted 1:10 with an autoclaved ultrapure water. All the processes carried out at this stage were done on ice. After the forward (forward - F) and reverse (reverse - R) primers for each gene (Synapsin-I, GAP43 and GAPDH (as a housekeeping gene)) are mixed and spinned with the help of vortex, 50 µl of each gene's F and R primers are taken and combined in an ependorph tube. Later, mixtures of SYBR green, ultrapure water, ROX (carboxy-X-rhodamine), dNTPs, Taq polymerase, $MgCl_2$ and pre-prepared gene primers at the rates specified in RT-PCR protocol were combined and “master mix” solutions were prepared for each gene separately. The result of the first process is 1:10 diluted 5 µl cDNA solution and 15 µl

“master mix” solution is mixed for each gene separately and RT-PCR device (Applied BioSystems) in a tube (3 separate tubes prepared for each gene). PCR analysis was performed by placing into RT-PCR device.

3. RESULTS

3.1. The Effect of Nerve Growth Factor (NGF) on PC12 Cells

To analyze the differentiation of nerve cells, the effects of commercially sold nerve growth factor on differentiation of PC12 cells were analyzed after solution and stocking. In other words, the NGF density is optimized to achieve the desired neural differentiation. Many studies in the literature have found that different methods and media combinations are preferred for the differentiation of PC12 cells. At this stage, different media combinations were also tested and the most suitable differentiation conditions were determined for the PC12 cells used. Basically, six different conditions were tested by combining two different media (serum media and serum-free media) and three different concentrations of NGF (0,100 and 200 ng / mL). Compared with serum medium, DHS level was decreased from 10% to 1% in serum-free medium and FBS was not used additionally (Figure 3.1).

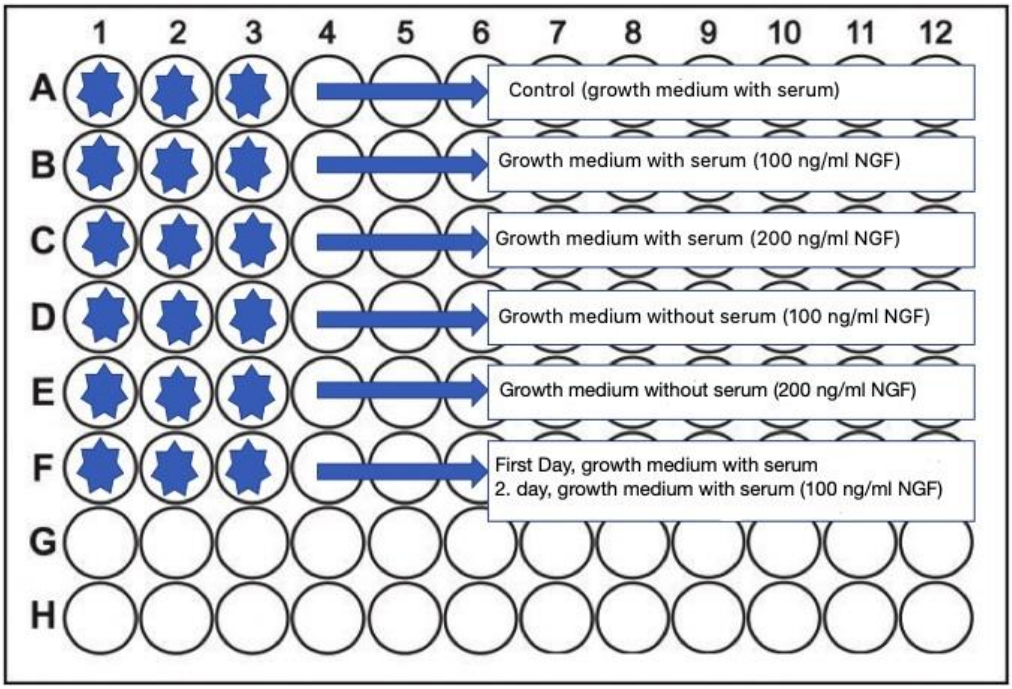

Figure 3.1 Experimental setup for the examination of their differentiation in growth medium containing high level of serum (RPMI 1640 + 10% DHS + 1% FBS + 1% L-Glutamine + 0.1% Gentamicin) and low level of serum (RPMI 1640 +%) 1 DHS + 1% L-Glutamine + 0.1% Gentamycin) with different concentrations of NGF.

Cells were examined daily under a microscope (CKX41, Olympus, Japan) and images were recorded to observe the results of the experiment, to see the effect of NGF and what medium to use.

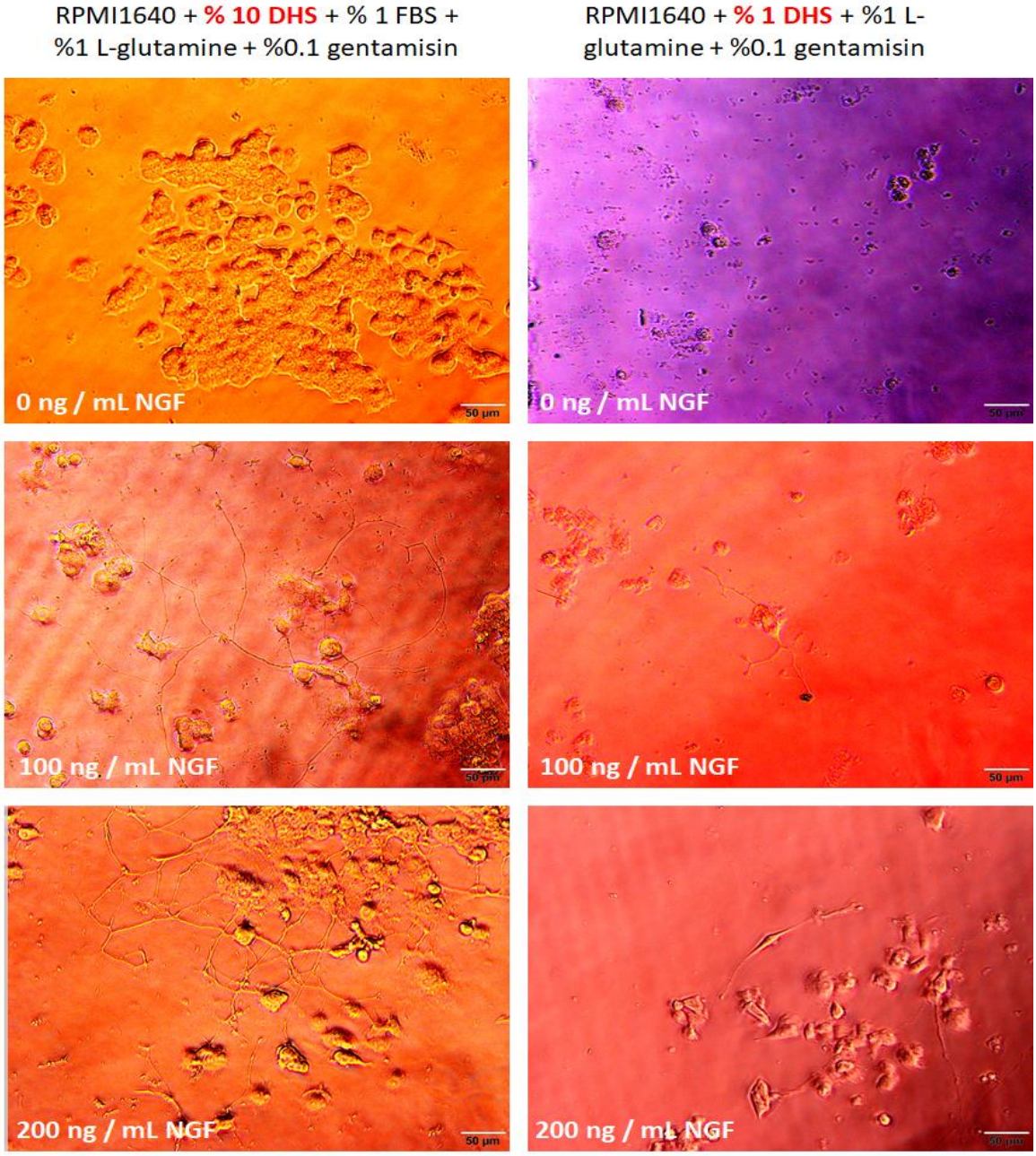

Figure 3.2 Microscope images of cellular differentiation on day six in the presence of 0, 100 and 200 ng / mL NGF in normal and low serum media of PC12 cells.

In some studies, in the literature, PC12 cells have been reported to show a better differentiation in medium containing NGF and low concentration serum [89]. Serum can contain a wide variety of growth factors such as differentiation factors,

catecholamines, which can lead to easily unidentified and unpredictable cell characteristics. Therefore, experiments with neural differentiation using PC12 cells are often performed after short-term culture in serum-free medium to exclude undetermined serum effects. As a result of the experiments, it was observed that the PC12 cells that we have were unable to maintain their viability at low serum density (Figure 3.2). Also, it was observed that only a small part of the cells differentiates in the presence of NGF. PC12 cells cultured on normal medium proliferated as expected (Figure 3.2). In the case of control without NGF, no protrusions, called neurites, which are also a symptom of differentiation, were found, whereas in the presence of NGF, the cells differed and long protrusions became evident. When NGF is used as 200 ng / mL, a slight difference compared to 100 ng / mL has been observed, but it has been concluded that 100 ng / mL NGF can trigger differentiation at the desired size. In fact, in conditions containing 100 ng / mL NGF in places, longer neurites were detected compared to the condition containing 200 ng / mL NGF (Figure 3.2). Comparing the degree of differentiation obtained in the presence of NGF in normal and low serum media, it was found that the cells differentiate much better in normal medium containing serum. In the light of the findings obtained, in the normal medium of differentiation of PC12 cells from now on (RPMI 1640 + 10% DHS (Donor Horse Serum) + 1% FBS (Fetal Bovine Serum) + 1% L-glutamine + 0.1% gentamicin) 100 ng / mL It was decided to be induced in the presence of NGF.

3.2 Effect of Electrical Stimulation on PC12 Cells onto ITO Microchips

In the literature, there are various studies on the effects of electrical stimulation in terms of neurite length, direction and number in differentiation of various neuron cells, including PC12. However, no study has been reported so far on exposing neuronal cells to electrical stimulation using IDA biochips and subsequently studying neural differentiation. Therefore, PC12 cells grown directly on ITO biochips for guidance in the later stages of the project were exposed to electrical stimulation at various times and the effects of electrical stimulation on neural differentiation in the presence of

NGF were compared in comparison with the control group without electrical stimulation.

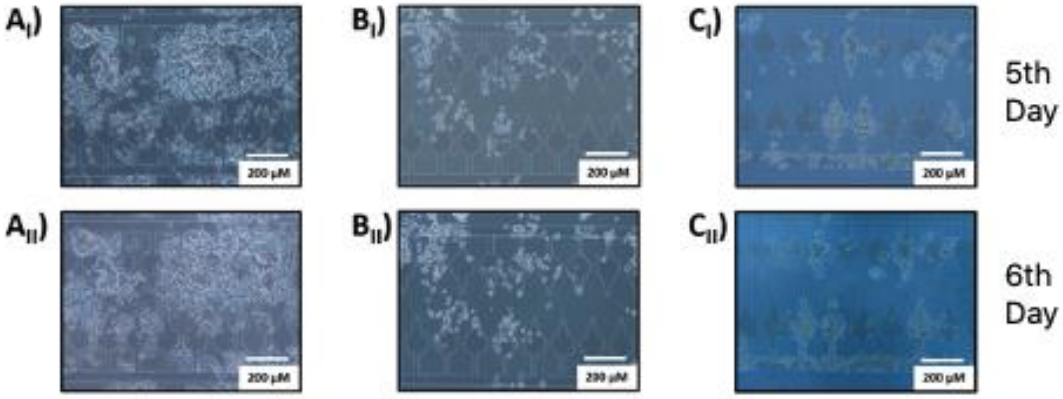

Figure 3.3 Pictures of PC12 cells grown without electrical stimulation on the biochip.

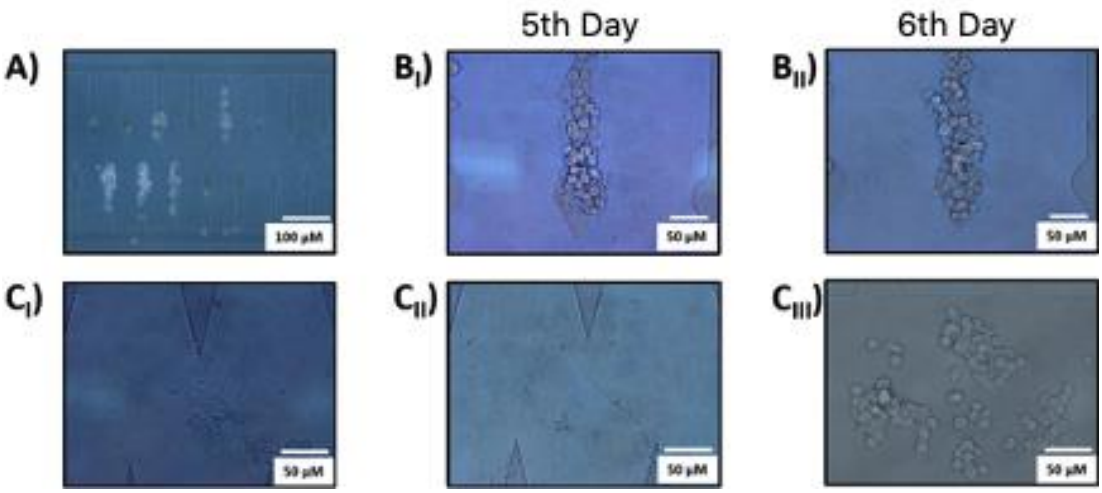

Figure 3.4 An image of PC12 cells grown in a different design and different period of electrical stimulation on the biochip.

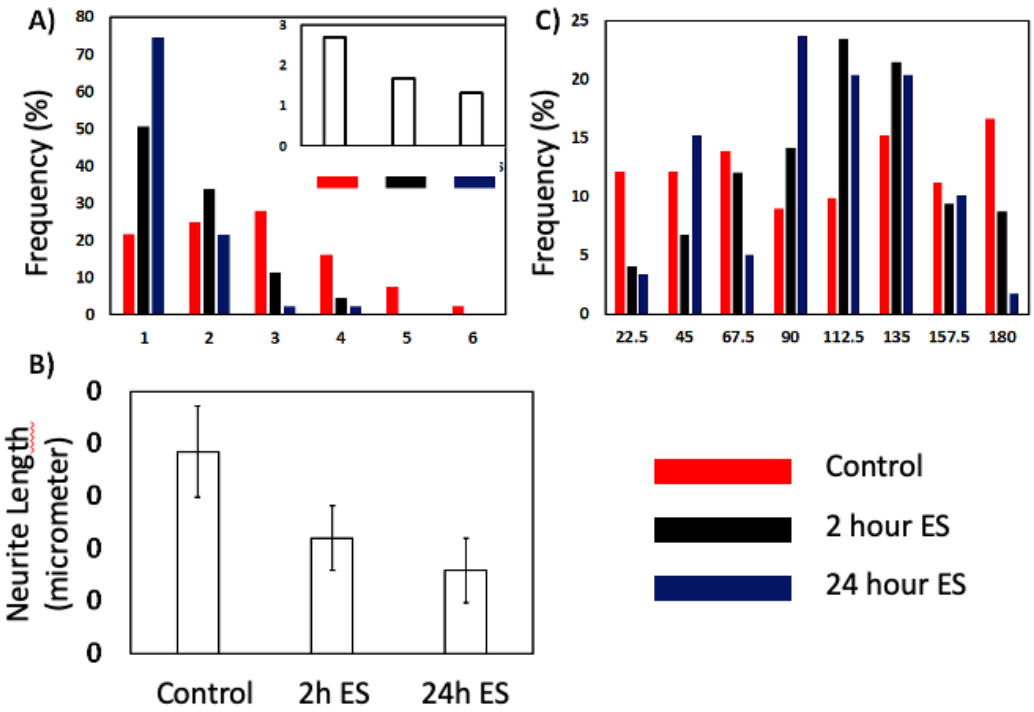

Figure 3.5. Average number of neurites per cell (A) and angular (C) orientation using the longest 30 neurites from the sixth day pictures of PC12 cells exposed to 2- and 24-hours electrical stimulation and control group PC12 cells without electrical stimulation. Analysis results showing neurite length (B).

Three different groups (biochips) were used for the experiment. The first group without electrical stimulation was used as a control. The medium was replaced with a medium containing NGF on the second day to induce neural differentiation in this group. For the second group, 100 mVpp / mm AC sine wave at 100 Hz frequency is 30 min. It was applied. In the third group, 100 mVpp / mm DC voltage is 30 minutes. Applied [87, 88]. After electrical application, the medium of all samples was replaced with medium containing NGF. As a result of the findings, it was observed that the cells grew randomly in the control group without electrical stimulation (Figure 3.3 AI-II), whereas in the test groups with electrical stimulation, the cells migrated towards the electrodes in relation to the duration of the electrical stimulation (Figure 3.3 BI-II-CI-II). As seen in Figure 3.3 CI – 3.4 A, BI, cells are concentrated on electrodes of different sizes and shapes. As seen in Figures 3.3 CII – 3.4 BII, it has been determined that this effect continues even 48 hours after electrical stimulation. As seen in Figure 3.4 BI-II, cells grew on electrodes in multiple layers (three-dimensional) rather than

single layer. Neurites belonging to PC12 cells in the control group did not prefer a certain direction as expected, but elongated randomly in each direction (Figure 3.4 CI-3.5 C). In PC12 cells in the test group where electrical stimulation occurred, a significant decrease was observed in the number and length of neurites per cell due to the duration of the stimulation (Figure 3.5 A-B), whereas the partial increase in the orientation of the cellular protrusions in relation to the applied electrical field was observed (Figure 3.4 CII-III- 3.5 C) . Many studies in the literature have reported that electrical stimulation at 100 mV / mm induces neurite outgrowth in PC12 cells. Considering the results obtained as a result of the experiments, it was concluded that the stimulation at the level of 100 mV / mm with biochip had a different effect than expected. According to the results obtained with the parameters used, it was determined that the cells themselves are directed towards the electrodes rather than the cellular protrusions, so it is concluded that these protrusions can be induced to extend in a certain direction with a lower level and short-term electrical stimulation. Considering the results obtained and the reports in the literature, 30 min. It was decided to apply electrical stimulation [87, 88].

3.3 Effect of Electrical Stimulation of PC12 Cell Differentiation on Graphene Coated ITO Microchips and Bare ITO Microchips

To study the effect of graphene on differentiation of nerve cells, the differentiation of PC12 cells was investigated using the commercially available nerve growth factor. 4 different groups were used for the experiment: ITO control and ITO microchip with direct current (DC) electrical stimulation, graphene coated ITO microchip and graphene coated ITO microchip with direct current electrical stimulation. PC12 cells were seeded on all these groups and NGF was added all groups and their microscope images shown (Figure 3.6). When comparing ITO control and ITO DC group, electrical stimulation caused the cells to orient and accumulate on the electrodes. In ITO DC group, differentiation was not really observed but it compares with graphene coated ITO with direct current electrical stimulation experiment, there are many neurite extensions. Neurite extensions was observed in control groups so cells could

be differentiated in every situation in the presence of NGF. For making new bridges between the cells, neurite lengths and neurite number per cell are important to obtain a good result. Considering these conditions, graphene coated microchips have more neurite lengths and number of neurites per cell so it might be said graphene accelerates the nerve differentiation and making new connections between cells due to nanostructure and electrically conductivity of graphene.

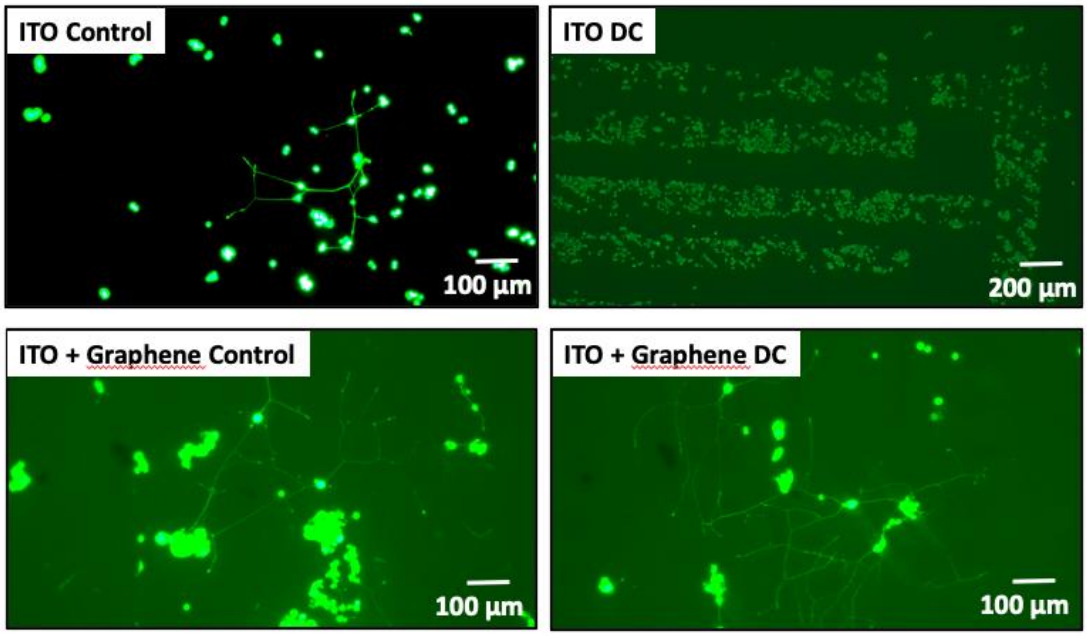

Figure 3.6 Pictures of PC12 cells grown with and without electrical stimulation on the ITO microchip and graphene coated ITO microchip from days 5. Electrical stimulation was applied only between 2 days in both conditions and cells have the same passage number when they are seeded on the microchip surface.

ITO microchip with direct current electrical stimulation group was observed day by day and behavior of cells was observed. In images of first, third, fifth and seventh day was taken from the same place of microchip under microscope (Figure 3.7). Direct current electrical stimulation provides to oriented the cells through the electrodes of microchip. When number of day increases, cells more oriented through the electrodes. It provides to make better connections between nerve cells by extensions.

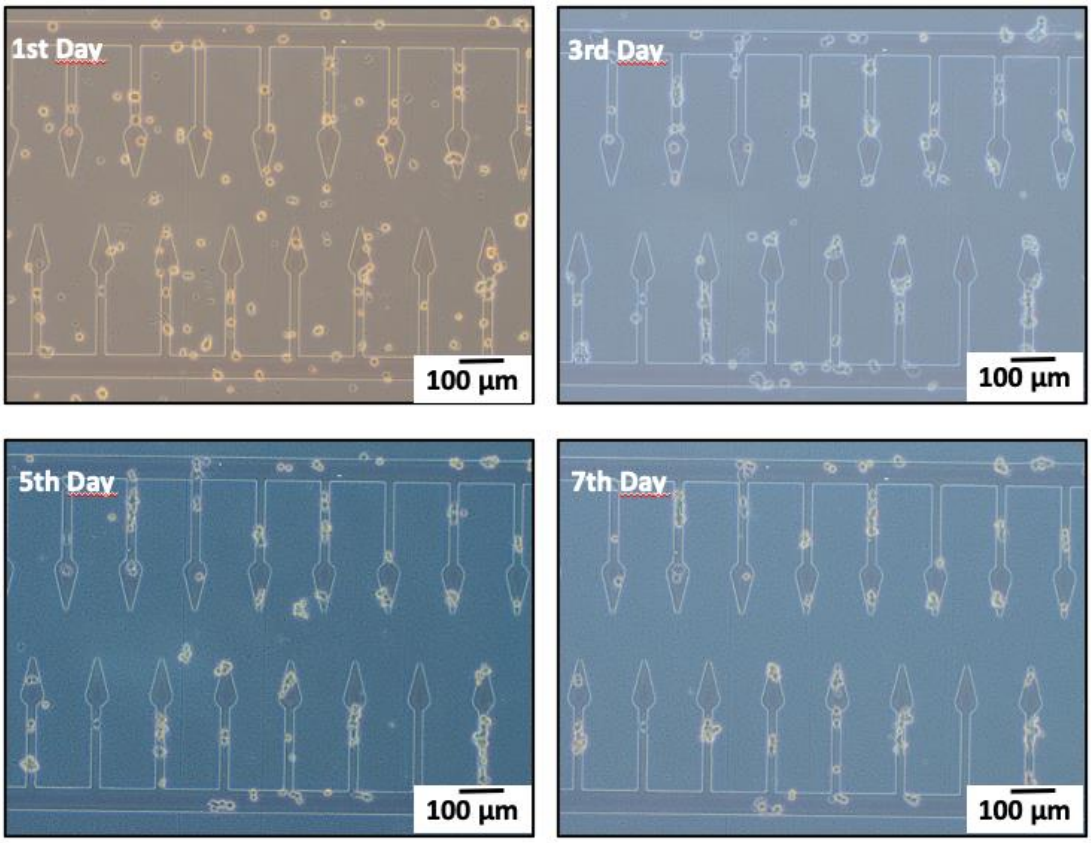

Figure 3.7 Pictures of PC12 cells grown with direct current electrical stimulation on the ITO microchip from days 1, 3, 5 and 7. Electrical stimulation was applied only 1 day the images were taken from the same place of microchip surface.

For evaluation of nerve differentiation clearly, some experiments are done with alternative and direct current electrical stimulation and without electrical stimulation on ITO microchip or graphene coated ITO microchip. All cells were seeded from the passage and all condition about nerve growth factor adding was the same and their images were taken under microscope and then images were analyzed by ImageJ program by measuring the neurite length and number of neurites per cell. All data becomes a graph for better understanding (Figure 3.7). When comparing all groups, the best neurite lengths are found in the graphene DC group. As before experiment showed, graphene provides more longer neurites according to ITO groups. ITO control groups have many neurites but they are not long enough. Graphene coated ITO microchip and graphene coated ITO microchip with alternative current showed nearly the same neurite number and length so alternative current is not working well. Graphene coated ITO microchip with direct current have the longest neurites as

average (A). Graphene coating is known as good for providing more longer neurites. When comparing ITO groups amongst themselves average neurite lengths are nearly same also neurite lengths of alternative current and direct current groups. Electrical stimulation on ITO microchips are found to have bad effect. ITO control group showed without electrical stimulation ITO microchip had a better effect on PC12 cells. Comparing control groups of ITO and graphene coated ITO microchip groups, graphene coated control group showed better effect as longer neurites as shown in before experiment. When graphene groups compared amongst themselves, graphene coated ITO microchip with direct current electrical stimulation showed more longer neurites because of their better orientation (B). Bare ITO groups compared amongst themselves, ITO control group have highest number of neurites per cells so electrical stimulation on bare ITO microchip has been shown to adversely affect. In all graphene groups, nearly the same number of neurites per cell but in direct current electrical stimulation on graphene coated ITO microchip has the highest number of neurites per cell. Electrical stimulation investigates the neurite outgrowth and number of neurites per cells on the graphene coated ITO microchip surface due to graphene's conductivity and nanostructure on microchips. (C). PC12 nerve cells tend to grow better on graphene and remove more neurites (Figure 3.8).

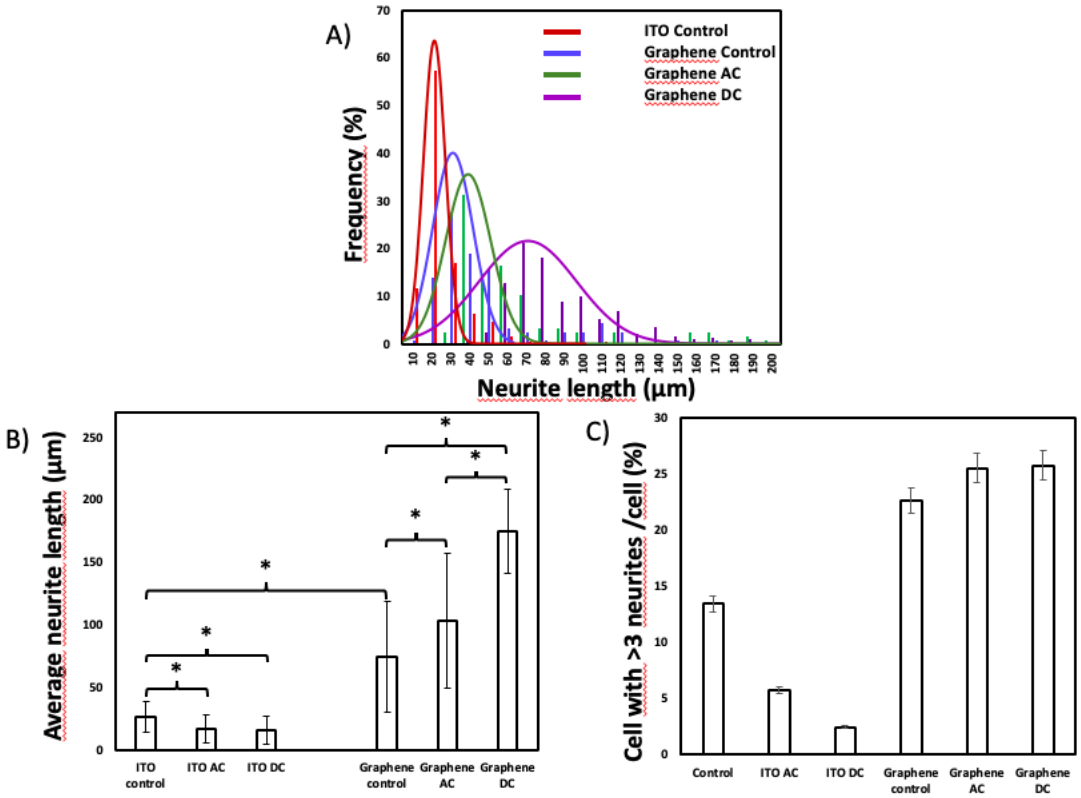

Figure 3.8 Pictures of PC12 cells grown with alternative and direct current electrical stimulation and without electrical stimulation on ITO microchip and graphene coated ITO microchip. All cells seeded from the same passage number and all conditions as nerve growth factor concentration and day of images are the same.

PCR (Polymerase Chain Reaction) is a process applied to gene sequence to replicate enzymatically in a certain region of DNA [90]. Differentiation of PC12 cells were obtained and showed by pictures but for the precision of data gene expression profile must be checked. Gene of differentiated cells are copied and their optimization were done by PCR method. In this experiment, SIN-1 (3-morpholinosydnonimine) and GAP43 (Growth Association Protein) genes are used for detecting the differentiation gene and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) gene is used for housekeeping gene and their primer (forward and reverse) sequences are given in the method section [91-94].

According to qPCR results, electrical stimulation on graphene coated IDA microchips according to graphene coated without electrical stimulation shows better differentiation activity from genes of differentiated PC12 cells (Figure 3.9). qPCR results are verified the results of previous data (Figure 3.8).

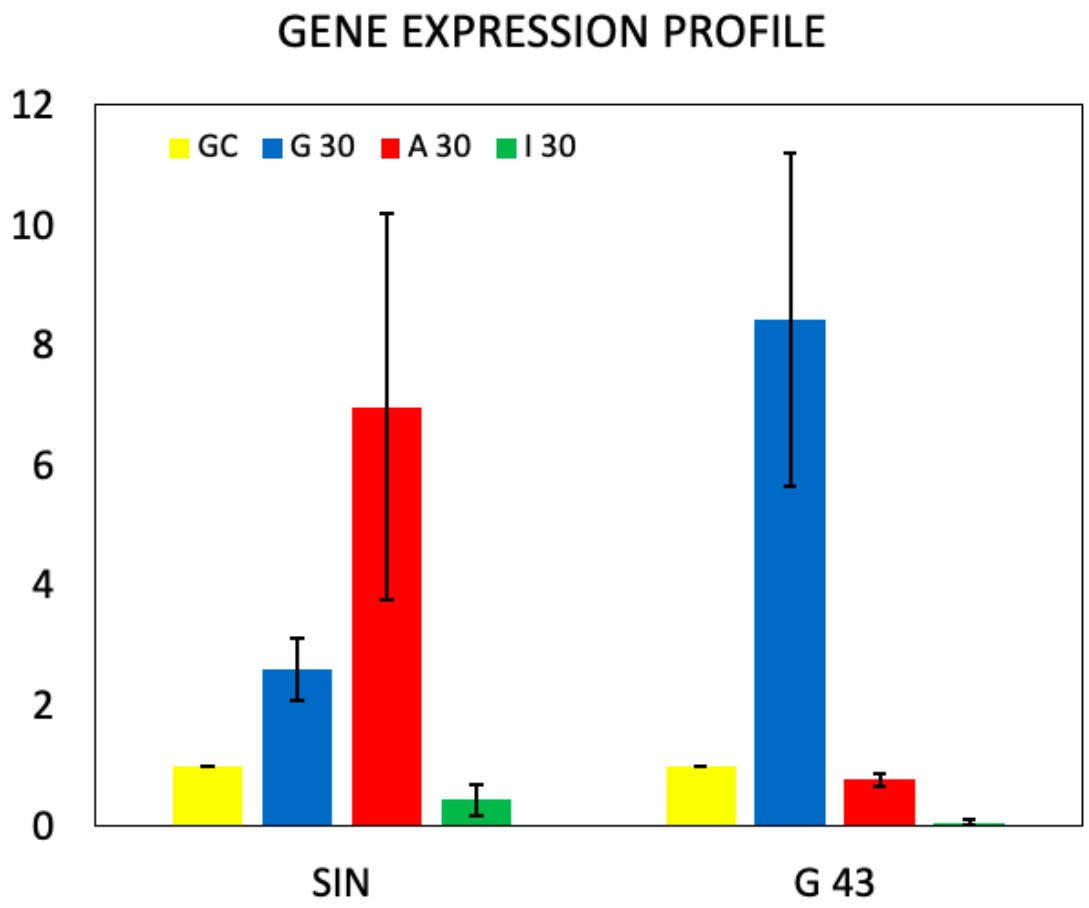

Figure 3.9 Graph has been obtained from qPCR results of PC12 cells are differentiated with NGF. (GC: Graphene coated ITO Microchip, G30: Graphene coated ITO microchip with direct current electrical stimulation applied for 30 min, A30: ITO microchip with alternative current electrical stimulation applied for 30 min, I30: ITO microchip with direct current electrical stimulation applied for 30 min.) SIN and G43 (GAP43) are genes that responsible for differentiation detection by using housekeeping gene GAPDH.

4. CONCLUSION

Most of the diseases that cannot be treated today are related to the nervous system and can be associated with the loss of the connection between nerve cells and the failure of nerve cell renewals. A neuronal network consists of connections between neuronal cells through dendrites and axons. Synapse formation between cells provides communication and signals can be received from these cells to investigate some nervous system diseases and treatments. The first step of forming such connections on a chip is to induce spreading of neuronal extensions. In this study, the impact of graphene on neuronal differentiation of PC12 cells into neuron-like cells was evaluated in conjunction with electrical stimulation. To apply the electrical stimulation, an ITO microchip was successfully fabricated using photolithography. Next, the concentration of NGF was optimized and 100 ng/ml was found to be sufficient to induce the neuronal differentiation of PC12 cells. When cells were induced to differentiate without electrical stimulation, they demonstrated a much better differentiation behavior on graphene coated ITO-microchips than bare microchips. Although both AC and DC electrical stimulation negatively affected neuronal differentiation on bare ITO microchips, they both demonstrated a significantly affirmative effect in inducing neuronal differentiation on graphene coated ITO-microchips. When the effects of AC and DC on graphene-coated ITO-microchips were compared, it was observed that the DC electrical stimulation applied in the presence of NGF for 30 minutes gave rise to the highest level of neuronal extension spreading. The positive effect of DC electrical stimulation was also verified with RT-qPCR test. In the future, the surface of graphene coated microchips will be patterned with a polymer to form physical barriers that will guide the spreading of neuronal extensions in a particular direction. The results of the study are expected to be useful in constructing neuronal circuits on a chip for various applications ranging from nerve regeneration to drug discovery.

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List of Publications:

- Seven, F., **Gölcez, T.** ve ŞEN, M. (2020). Nanoporous carbon-fiber microelectrodes for sensitive detection of H2O2 and dopamine. *Journal of Electroanalytical Chemistry*, 114104. **(Impact Factor: 3.807)**
- Seven, F., **Gölcez, T.**, Yaralı, Z. B., Onak, G., Karaman, O. ve Şen, M. (2020). Guiding neural extensions of PC12 cells on carbon nanotube tracks dielectrophoretically formed in poly (ethylene glycol) dimethacrylate. *RSC Advances*, *10*(44), 26120-26125**. (Impact factor: 3.119)**
- **Golcez, T.,** Kilic, V. ve Sen, M. (2020). A Portable Smartphone-Based Platform with an Offline Image Processing Tool for Rapid Paper-Based Colorimetric Detection of Glucose in Artificial Saliva. **(submitted)**
- Seven, F., **Gölcez, T.,** Şahinler, M., Şendemir, A., Karaman, O. ve Şen, M. (2019, October). Prolonged Electrical Stimulation of Neuronal PC12 Cells Using a Microchip. In *2019 Medical Technologies Congress (TIPTEKNO)* (pp. 1-4). IEEE.
- **Gölcez, T.,** Kiliç, V. ve Şen, M. (2019, October). Integration of a Smartphone Application with a ¼PAD for Rapid Colorimetric Detection of Glucose. In *2019 Medical Technologies Congress (TIPTEKNO)* (pp. 1-4). IEEE.
- Seven, F., **Gölcez, T**., Yaralı, Z. B., Karaman, O., & Şen, M. (2020) Guiding neural extensions of PC12 cells on dielectrophoretically aligned MWCNTs in poly(ethylene glycol)dimethacrylate. *EurasianBioChem*
- **Gölcez, T**., Seven, F., Ersü, G., Güneş, F., Yaralı, Z. B., Karaman, O., & Şen, M. (2020) Electrical stimulation of PC12 cells for neural differentiation on monolayer graphene coated IDA-microchips. *EurasianBioChem 2020*
- Seven, F., **Gölcez, T**., & Şen, M. (2020) High-sensitive detection of H2O2 and dopamine using thermally annealed carbon-fiber microelectrodes. *EurasianBioChem 2020*

Project:

- Fabrication of a lab-on-a-chip device for neuronal guiding. (Supplied by TUBITAK), 2018-May 2020 (Project No: 215E003).

Certificates:

- Training of the development of vocational qualifications in the fields of biomedical calibration and technology by IZKA - Izmir Development Agency (TR31/13/OME02/0135)
- Clinical ionized radiation safety training (BIOMUT 2016)
- System and software solution training in biomedical engineering-4C medical (BIOMUT 2016) -ISO 9001:2015 Training of Quality Management System
- GMP: Good Manufacturing Practice
- GLP: Good Laboratory Practice
- ISO 13485:2016 Medical Devices Quality Management System
- ISO 45001:2018 (New) Occupational Health and Safety Management System