Neuronal sensing via inkjet printing of biocompatible graphene

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Neuronal sensing via inkjet printing of biocompatible graphene

by

Jingshuai Guo

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Mechanical Engineering

Program of Study Committee:
Nicole Nastaran Hashemi, Major Professor
Reza Montazami
Chenxu Yu

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2019

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DEDICATION

I humbly dedicate this thesis to my parents and my fiancée. I am grateful for being given the resources and encouragement to pursue my education, and unwavering beliefs you always had in me.
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This work was partially supported by the Office of Naval Research Grant N000141712620 and the Iowa State University Department of Mechanical Engineering. I thank Dr. Anumantha Kanthasamy of Department of Biomedical Sciences at the Iowa State University for the gift of Rat dopaminergic neural cells (N27s).
ABSTRACT

Parkinson’s diseases (PD) has become the second most common neurodegenerative disease in the United States of America. To study how PD bioelectric properties acts, an easy application, unexpansive graphene-based biosensor design has been introduced. This thesis demonstrates how to set up this biosensor, inkjet printing few-layer graphene (FLG) ink on to Kapton Polyimides (PI) and applied Cell Live-Dead Assays to prove sensor biocompatibility. This study helps us move forward to the N27 cell lines bioelectrical signal studies for our next study.
CHAPTER 1. INTRODUCTION

1.1. Overview

The diagnosis of Parkinson’s disease (PD) is based on the identifications of some combinations of bradykinesia, rigidity, tremors, and postural instability. (1) It is a progressive, age-associated neurodegenerative disorder caused by loss of dopaminergic neurons of the substantia nigra (SN); it is the second most common neurodegenerative disease in the United States after Alzheimer’s. (2,3) To fully understand PD, a vitro model, N27 cell line, has been developed. This cell line is based on embryonic rat mesencephalic dopamine neurons immortalized with SV40 large T antigen. (4) Studying the electrophysiology of this cell line could help us understand more on how neuronal cells’ electro behaviors can lead us to cure Parkinson’s disease in the future.

In 2004, graphene was discovered. This new material captured the attention of material engineers and material scientists. A few graphene properties are listed below:

1.1.1. High Electron Mobility

Graphene has a zero-band gap semiconductor feature which has an ambipolar electric field effect. Charge carries can be tuned continuously between electrons and holes in concentrations as high as $10^{13}$ cm$^{-2}$, and under room temperature, this number can be raised by up to 15000 cm$^{2}$V$^{-1}$s$^{-1}$. (5)

1.1.2. High Elasticity

From studying graphene’s Young’s modulus and fracture strength, for defect-free graphene, a Young’s modulus of 1.0 TPa and a fracture strength of 130 GPa were found; the
reduced graphene oxide has a mean elastic modulus of 0.25 TPa with a standard deviation of 0.15 TPa.(6)

1.1.3. High Transmittance

Graphene has been stated to have a constant high-frequency conductivity of 2.3% for Dirac fermions, from infrared through the visible range of the spectrum.(7,8)

1.1.4. High Thermal Conductivity

Graphene has a diffusive conduction at high temperatures and a ballistic conduction at low temperatures due to its relatively low carrier density and negligible electronic contributions.(9) At room temperature, the monolayer graphene has a thermal conductivity of about 6000 Wm⁻¹K⁻¹.(10)

1.2. Graphene Production Methods

In 2004, graphene was discovered by Novoselov et al, and since then, multiple manufacturing processes of graphene have been developed. A few majority methods that have been used by the industry during research are described below:

1.2.1. Chemical Vapor Deposition

Chemical Vapor Deposition (CVD) has been developed over the last 40 years. This method is based on depositing layers of nickel of 300 nm thickness on to SiO₂/Si substrates using an electron-beam evaporator. Then the samples are heated up to 1000 °C inside a quartz tube under an argon atmosphere and cooled down to room temperature using flowing argon. This provides an efficient method of separating graphene layers from the substrate. The minimum sheet resistance of CVD graphene can reach 280 Ω per square.(11)
1.2.2. Liquid-Phase Exfoliation

This is a method of graphite dispersion and exfoliation in organic solvents such as N-methyl-pyrrolidone. This method involves using the energy balances between exfoliate graphene and solvent-graphene interactions. This method could be used to deposit graphene in many environments. (12)

1.2.3. Mechanical Exfoliation

This is a simple and efficient method developed by Novoselov and Geim. Adhesive tape is repeatedly stuck to and peeled from graphite, which brings a 1 μm thick graphite flake to a monolayer-thin sample, which is then further thinned with tape-to-tape peeling. Graphene is eventually ready to peel onto SiO₂/Si substrate along with many smaller crystals on the tape. Some of those smaller crystals are single-layer graphene.

Because the carbon layers are weakly bonded, the van der Waals force between layers is not as strong as the force between the graphite/graphene and the SiO₂. Since the bonds in between graphite/graphene are easy to break, the graphite pieces on the tape that are applied to the SiO₂ substrate are most likely leaving some amounts of graphite/graphene behind on the substrate. (13) Further Raman spectra results show prominent G and 2D peaks for the newly peeled single-layer graphene.

The new mechanical exfoliation methods involve using more efficient equipment rather than using adhesive tape continuously peeling graphite. Equipment such as ball milling and a kitchen blender were used for higher scale graphene exfoliation productions. (14,15)
1.2.4. Biocompatible Graphene (BSA)

For a cell-friendly approach, bovine serum albumin (BSA) has been used for graphene production. This exfoliation method results in hydrophobic and hydrophilic patches on the BSA surface which are easily switched. Based on electrostatic adherence, the negatively charged NPs remain on the BSA-graphene bio interface. This method is normally followed by strong physical forces (e.g., shaking or ball milling) to further break down the graphite particles.(14,16,17)

1.3. Research Motivations & Challenges

After many advanced manufacturing methods of producing graphene for commercial purposes, there is one application that benefits the most in studying Parkinson’s disease. The excellent electrical conductivity of graphene is a perfect fit for studying the electrophysiology of the N27 cell line since this is a vitro model of PD. There are many studies on how graphene-based biosensors are applied; Sahil Rastogi et al. studied how graphene effects nonneuronal and neuronal cell stress and viability, proving that CVD graphene could be biocompatible. Further studies show when using graphene based electrodes for cell sensing, graphene electrodes have strong flexibility in their electrochemical properties.(18,19) L. Wu et al. provided a concept of Plasmon resonance; however, this idea was never proved.(20) Rui Wang et al introduced ultrasensitive graphene optoelectronic probes for recording electrical activities; however, the chip design requires a four-chamber neuron glia coculture microfluidic device with an integrated graphene transistor design, which is a very complicated device.(21)

To make an easy application unexpansive graphene-based biosensor chip in this study becomes necessary. This would help us to understand how electrophysiology effects N27
cells. The following chapters describe an easy application of BSA-graphene based neuronal cell sensors using custom built inkjet printers for printing onto Kapton Polyimide (PI) substrate.

The challenges of this research were frustrating at the beginning. Serval trials of the graphene wet ball milling method were conducted; however, the end results were not promising. Initially, the method was to use water sonication and the stirring of BSA and graphite to produce graphene. Unfortunately, this method requires continuous switching between the sonication and mixer for 36 hours. Literature applied to this method included using a combined sonication and mixer machine to complete this task, but our lab does not contain this kind of equipment.

During the printing process, even though our set up was easy to apply, due to the limitation of our USB CNC machine, G-coding could not be applied while using the CNC to print. Manual control took the place for all the prints presented below. The end results for the prints were good enough to present in this thesis, however, in the future, this issue needs to be fixed.
CHAPTER 2. INKJET PRINTED GRAPHENE ON POLYIMIDE FOR SENSING NEURONAL CELLS

Inkjet Printed Graphene on Polyimide for Sensing Neuronal Cells

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2.1. Abstract

The second most common neurodegenerative disease in the United States after Alzheimer’s disease (AD) is the Parkinson’s disease (PD). To help understand how electrophysiology effects these diseases, an N27 cell line was applied as a vitro model. In this study, a flexible graphene-based biosensor design is demonstrated. Biocompatible graphene was made through a liquid-phased exfoliation method using Bovine Serum Albumin (BSA). Raman spectroscopy results indicates that the graphene produced is few layer graphene (FLG) with \((I_D/I_G)_{\text{Graphene}} = 0.11\). Inkjet printing of this few layer graphene (FLG) ink on Kapton Polyimides (PI) followed with scanning electron microscopy (SEM) for graphene prints characterizations shows an average width of \(\approx 868 \mu \text{m}\) with a normal thickness of \(\approx 5.20 \mu \text{m}\). Neuronal cells were placed on thermally annealed printed graphene chip. Live-dead cell assays were applied for proving sensor biocompatibility. A cell viability of approximately 80% was observed over 96 hours, which indicates that annealed graphene on Kapton PI substrate could be used as a neuronal cell biosensor. This research will help us move forward with studying N27 cell line biological electrophysiology signals.
2.2. Introduction

Parkinson’s disease (PD) is a neuronal disease that caused by the death of dopaminergic neurons in the substantia nigra pars compacta. It is currently the second most common neurodegenerative disease in the United States after the Alzheimer’s disease (AD). Because of their dopaminergic properties, Rat dopaminergic N27 cells have been widely used in *in vitro* models for PD studies, as well as studies seeking to understand problems such as neurotoxicity, oxidative stress, histone deacetylase (HDAC), and other molecular pathways. While there have been studies on the synaptophysin signaling of N27 cells, the electrophysiological effects of graphene on N27 cells are currently unknown. Since a study on the electrophysiology of N27 cells is necessary to further understand PD and other neurodegenerative diseases, graphene may be helpful in better understanding neurodegenerative diseases by serving as a useful biosensor.

Graphene is among the most widely used materials in the field of material science. Graphene has a two-dimensional honeycomb nanostructure with one-atom-thick planar sheet, sp²-bonded carbon atoms. Graphene has a large theoretical specific surface area of 2630 m² g⁻¹; intrinsic mobility of 200,000 cm² V⁻¹ s⁻¹, and superior thermal conductivity of approximately 5000 W m⁻¹ K⁻¹. Since it also has excellent electrical conductivity, it lends itself to many real-world applications and provides an ideal base for bioelectronics and biosensing.

Although there are many ways to produce graphene, the end products are not biocompatible. For examples, popular graphene solutions such as dimethylformamide (DMF) and N-methylpyrrolidone (NMP) are toxic and often result in a low-concentration graphene solution. Therefore, it is necessary to develop a cell-friendly approach for
large-scale production of biocompatible graphene. To address this problem, research on the synthesis of graphene-based solutions via oxidation (modified Hummer’s method) and chemical vapor deposition (CVD) method were investigated. However, there is evidence showing that Hummer’s method creates single carbon atom defects along with nanosized cracks. CVD works well for producing large continuous films of graphene but this method has been shown to result in numerous surface voids and defects, and there has been very limited success incorporating this method into desired applications. As the process of researching biocompatible graphene and practical applications such as printing graphene continues, very few studies have been able to produce favorable results. Fortunately, the method of direct Liquid-Phase Exfoliation (LPE) of graphite into graphene has been reported as an attractive approach for inkjet printing and cell-based studies.

There have been many previous studies on constructing biosensors using graphene-based material. One such example involved using graphene oxide (GO) to examine cellular DNA via the creation of graphene quantum dots (GQDs). While this technique was used to image cancer-stem cell, the biosensors in these studies were primarily focused on the transfer of electrons using cyclic-voltammetry (CV) techniques. For example, while \([\text{Fe(CN)}_6]^{3-/4-}\) and \([\text{Ru(NH}_3)_6]^{3+/2+}\) redox peaks have been measured, a study on bioelectrical signaling of neuronal cells using graphene-based sensors is still lacking. One advantage of using graphene in biosensor study is that this material has no effect on mitochondrial membrane potential (MMP), and also has no effect on mitochondrial morphology or cell stress. Since graphene appears to have a bright future in biochemical and biomedical applications, there is a push for engineers to continue research studies on this material.
In this study, demonstration of a prototype graphene biosensor for the electrical signaling of N27 has been presented. The biosensors were produced with an inkjet printer to create conductive, biocompatible, and defect-free graphene. The graphene was exfoliated from graphite using the Liquid-Phase Exfoliation (LPE) method. This design presents a facile technique that can be used to manufacture biosensors for a variety of applications. In a previous study, Bovine Serum Albumin (BSA) was used to exfoliate graphene in an aqueous state that exhibited strong biocompatibility. Since it was manufactured with ease, it will lend itself nicely to large-scale production and hence this process was adopted for this study. The graphene and printed graphene chips were characterized using Raman spectroscopy, Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM). To characterize cell viability within the biosensors, multiple live-dead cell assays were performed.

2.3. Materials and Experimental Section

2.3.1. Materials and Equipment

The materials and equipment used in this study were: graphite (Synthetic powder, <20 μm, Sigma-Aldrich, St. Louis, MO); Albumin, from Bovine Serum (Bovine albumin* BSA, ≥98% agarose gel electrophoresis, lyophilized powder, Sigma-Aldrich, St. Louis, MO); Poly(sodium 4-styrenesulfonate) (PSS) (Mw ~ 1,000,000 powder, Sigma-Aldrich, St. Louis, MO); Poly((ethyleneimine), solution (PEI) (50% w/v, Sigma-Aldrich, St. Louis, MO); Sodium Chloride (NaCl) purchased from Sigma-Aldrich, St. Louis, MO; Kapton Polylimide (PI) (Thickness: 0.008mm, 100×100 mm, Sigma-Aldrich, St. Louis, MO). RPMI Medium 1640 (1X) (Ref#: 11875-093, 500mL), L-Glutamine 200mM(100X) (Ref#: 25030-081, 100mL), Pen Strep ((10,000 U mL⁻¹)-streptomycin (10,000 μg mL⁻¹) Ref#: 15140-122, 100mL) were purchased from Gibco Life Technologies. Fetal Bovine Serum (FBS) (Qualified One Shot™,
Ref#: A31606-01, 50mL) was purchased from ThermoFisher Scientific, Waltham, MA. Polyurethane Ether tube (I.D.:0.063”, Wall:0.031”, O.D.:0.125”, Part Number: 2100070-100) was purchased from Superthane®. 3mL Luer-Lok Syringes were purchased from AllegroMedical.Inc. Six-Well Cell Culture Clusters (Lot# 23314037) were purchased from Costar®. A GenieTouch™ Syringe pump was purchased from Kent Scientific Corporation. A 4 axis CNC USB controller Mk3/4 for Mini CNC mill was purchased and controlled by a PlanetCNC®. A Sinometer Digital Multimeter (MS8261) was used to measure print conductivity. A JEOL FESM JCM-6000 Scanning Electron Microscope and a Zeiss Axio Observer Z1 Inverted Microscope were used for SEM imaging of the graphene prints and the Live-Dead Cell Assay. A Raman Spectrometer (Voyage, B&W Tek, Inc.) with a CW laser (Excelsior-532-150-CDRH, Spectra-Physics) was used for Raman Spectroscopy measurement.

2.3.2. Preparation of Graphene

The graphene solution used in this study was produced by the liquid exfoliation method, which involved using wet-ball milling method by a Vibrio-Energy shaker mill(53,54) and blender mixing method.(14) This is due to the similar cartelization results from both samples’ Raman spectroscopy results (Figure 2-13). The shear tension created by the steel balls helps the exfoliation of graphite crystallites thus fabrication of high-quality few-layer graphene (FLG).(28,55,56) 650 mg of graphite was mixed with 60 mg of BSA and well-dispersed in 35 mL of DI-H₂O. A Vibro-Energy Mill (Shaker Mill) was run for 90 hours at 300 rpm (Figure 2-1). 20 steel balls of diameters of 8.7 mm (11/32”) and 10 steel balls with diameters of 4.5mm (3/16”) were added to all five containers (Figure 2-3). For the blender method, a standard kitchen blender was run for 1 hour at speed of 16761 rpm. 20 g of graphite mixed
with 605 mg of BSA well-dispersed in 100 mL of DI-H$_2$O. Both graphene solutions were kept at rest for 24 hours, allowing any remnant of non-dispersed graphite particles to sink to the bottom of the containers.

Figure 2-1. Shaker mill for solution production

Figure 2-2. Wet ball milling container setup
2.3.3. Substrate Preparation

Because of its great thermal resistance, Kapton PI was chosen as the polymer substrate, since it can withstand temperatures from -269 to 400 °C. Due to the FLG ink is hydrophilic and negatively charged, Kapton PI’s hydrophilicity treatment is necessary. To treat the Kapton PI, the PI was first washed in acetone. The substrate was next washed with PSS (wt. 13.5 mg/mL), submerged in a solution of 50 mL of DI-H₂O and NaCl (0.5 mol L⁻¹), and finally submerged into a PEI (wt. 30 mg/mL) of DI-H₂O and NaCl solution (0.5 mol L⁻¹) for another twenty minutes. The substrate was then allowed to air dry for 12 hours.

2.3.4. Inkjet Printing Procedure

Graphene ink was placed in a 5 mL syringe, attached with a 10cm long Polyurethane Ether tube. The graphene ink was injected through a hypodermic needle with an inner diameter of 300 µm. The syringe was fixed, and the graphene ink was pushed by a syringe pump to introduce at a rate of 3 µL s⁻¹, with the PI substrate fixed on a 22×22 mm glass cover chip. The chip was covered with two pieces of copper tape with a 0.50 mm gap between them.
(Figure 2-5). Figure 2-4 shows the general schematic of the printer and chip setup. A 3-kV potential difference (Figure 2-8) was applied between the needle and the substrate, with the needle position controlled by the CNC mini mill and its associated software. Prints were manufactured with a space of 800 µm in between each print, and a total of 5 graphene lines were printed on one chip. Figure 2-6 shows the final printing result after a heat treatment. A total of 6 graphene chips were made during each printing session.

**Figure 2-4. Schematic of printer setup**
Figure 2-5. Schematic of chip setup

Figure 2-6. Printing results
Figure 2-7. Equipment setup during prints. A syringe pump was used along with an inkjet printer to deposit ink at a rate of 7 µL/s to ensure a constant flow rate throughout the syringe.

Figure 2-8. Needle and substrate setup. A 3 kV potential differences were introduced between the substrate and needle for the purpose of affixing ink on to the substrate. Cover glass was placed on a regular microscope slide for stability and support.
2.3.5. Post Processing and Conductivity Testing

Post-processing was used for improvement of conductivity and stability of the printed graphene.(57,58) A preheated standard oven with setting temperature of 280 °C was used in an annealing process. After thirty minutes of annealing, the graphene chips were gently placed in a clear six-well-plate. A sterilization process for all chips were required, which allows the cells to safely grow on the top of prepared chips. A digital multimeter as used for conductivity testing, and the resistance of each line was measured every 3 mm across the gap created on the chip. The width of each line was then measured using a Scanning Electron Microscope (SEM) and found to be $868 \pm 20 \mu m$; the height of each line measured by the SEM was $4 \pm 1 \mu m$. The conductivity of each line could then be calculated from Equation (1), where $R$ is the resistance of a printed line, $l$ is its measured length, $w$ is its width, and $\sigma$ is the conductivity.(59,60)

$$R = \frac{l}{wt\sigma} \quad (1)$$

2.3.6. Raman Spectroscopy

A thin film of graphene sample was prepared to determine the Raman spectra. A drop of graphene sample of diameter approximately 10 mm was dropped on top of a Si/SiO$_2$ substrate was air-dried. A Raman Spectrometer with a CW laser provided a laser beam of wavelength 532 nm to the graphene on the Si/SiO$_2$ sample. Figure 2-10 depicts the Raman results for the graphene sample, on which 5 points were acquired by the Raman spectra.
Figure 2-9. Sample area image under direct 532nm laser, no cracks on the sample.

Figure 2-10. Raman spectra of graphene on Si/SiO2 under 532 nm laser.
2.3.7. SEM Imaging

Imaging of the printed graphene patterns on PI was performed in a SEM using a 2-5 kV accelerating voltage. The thickness is shown in Figure 2-11 to Figure 2-12 that depicts images of prints before and after annealing.

![SEM Image](image1.png)

Figure 2-11. SEM images of printed graphene on PI using 2-5kV accelerating voltage. SEM top view image for after annealing prints, which shows a normal width of ≈ 868 µm

![SEM Image](image2.png)

Figure 2-12. Prints cross section images, average thickness is approximately 5.20 µm

2.3.8. Chip Biocompatibility Tests

A flask of 5-time passaged N27 cells was chosen for testing graphene sensor biocompatibility. Graphene chips with six-well plates were placed under UV light inside a biologic fume hood for over 12 hours for sterilization before applying cells. In this study,
N27s cells were cultured in 3mL of maintenance media (MM) that includes RPMI Medium 1640 (1X), 10% FBS, 1% penicillin, and 1% L-Glutamine. 3 mL medium was then fulfilled in each well-covering graphene chip, and N27s with a cell density of ≥1×10⁶ cells/vial was dropped onto to the chips using a micro pipit in 10 µL amounts until all prints were covered. Cells were left to grow in an incubator maintained in a 37°C, 5% CO₂ atmosphere, and were checked under inverted microscopy at 24 hours, 48 hours, and 96 hours.

2.3.9. Live-Dead Cell Assay

Live-dead cell assays were performed using a 10 µM CellTracker™ CMFDA solution combined with and 8 µM propidium iodide (PI) in FBS-free RPMI media. MM was carefully removed from the desired well of the 6-well plate, and after removal from the well it was rinsed with FBS-free RPMI media (500 µL). 500 µL of dye was added to the well and the cells then incubated for 30 minutes at 37°C in a 5% CO₂ atmosphere. The dye was then removed and FBS-free RPMI media was added to keep maintain sample moisture for imaging. As shown in the control well (Figure 2-20), live cells were colored in green and dead cells were colored in red.

2.4. Results and Discussions

2.4.1. Chip Designs

Traditional graphene-based biosensors are expensive and are still in a conceptual stage. One fabrication method is to synthesize graphene on a glass slip and etch SiO2 with a Si/285nm laser,(18) another uses surface plasmon resonance to fabricate graphene on a gold sensor to achieve higher sensitivity than a tradition gold thin film SPR sensor ((1+0.025 L) × Ŷ (Ŷ>1)).(20)
In this design, the graphene biosensor chip included three components: glass coverslip, copper tape, and a polyimide (Kapton) polymer substrate (Figure 2-5). The copper tape was affixed on both sides of the chip with a 0.5 mm gap and covered by Kapton PI to fully covering the copper tape and prevent any exposure to the cells and any possible cytotoxicity (61). Since the gap between the copper tape is \( \leq 0.5 \text{ mm} \), the Kapton PI substrate was fully expanded to connect the both end with a flat surface thus the effect of this gap on the 3D prints is negligible (Figure 2-6).(62,63) This design ensures that light can penetrate through the gap between the copper tape layers and reduces damage to the prints, offering an inexpensive physical design that is ready to use.

2.4.2. Substrate Hydrophilicity Treatment & Inkjet Printing of Graphene

Aqueous graphene was pre-prepared using wet ball milling technology as described previously.(53) This paper focuses on graphene printing and sensor preparation. Graphene ink was applied to Kapton PolyIimide (PI), using the custom-designed electronic inkjet printer represented in Figure 2-4. Because the FLG ink is hydrophilic and negatively charged,(64) the PI substrate required some surface modification before successful printing could take place; the substrate was separately submerged into two different wetting agents: PSS and PEI, producing a hydrophilic layer on PI. This process created a hydrophilic buffer layer and changed the substrate from hydrophobic to hydrophilic.(65) A review of literature disclosed that traditional inkjet printing is using a commercial printer that does not support high viscosity ink, negatively affecting print quality.(41,66) In this setup, graphene ink was directly pushed out through a needle and printed on a PI substrate under force of an electrical field. We have found that printing at a flow rate of 3 \( \mu \text{L/min} \), using a needle of inner diameter 300 \( \mu \text{m} \) and a 3kV voltage produces stably continuous graphene with equal width and
thickness (Figure 2-13). The rather high value of electrostatic field was necessary to fix the conductive ink (67).

Figure 2-13. Microscope image of printing result presents an area of prints under microscope. Red, blue and green arrows are for graphene, copper tape and PI after annealing process.

To validate graphene quality, Raman Spectroscopy of a graphene drop on SiO$_2$ was performed using a 532 nm laser wavelength (Figure 2-9). A couple of details can be observed from Raman characterization. From previous studies (53, 54), the acquired spectrum experienced a sharp G peak at ~1569.19 cm$^{-1}$, a symmetrical 2D peak at ~2689.55 cm$^{-1}$, and a peak of D at ~1348.33 cm$^{-1}$ indicating that our graphene was few layer (68). Furthermore, the calculated ($I_D/I_G$)$_{graphene}$ of blender mixed graphene is equal to 0.11, and 0.16 for wet ball milled graphene. To further attempt characterization of printed graphene, a scanning electron microscope (SEM) was performed, producing SEM images (Figure 2-12) that confirm that graphene prints have widths that average of 868 µm with no voids, confirming the purity of our graphene prints that would avoid adverse effects of biological interaction.
Additionally, Atomic Force Microscope was also applied for graphene sample thickness characterization (Figure 2-14 to Figure 2-16).

Figure 2-14. Graphene flake

Figure 2-15. AFM tapping frequency.
2.4.3. Post Treatment of Graphene Prints

After printing process has been finished, a thermal annealing process also been applied to the complete prints. This is for further disrupting the conductive network due to the traditional solvents and surfactants employed for graphene exfoliation remain persistent residues. An oven was pre-heated to 280 °C for the thermal treatment of printed graphene. Graphene printed chips were annealed for 30 minutes before being removed for conductivity measurements. There are evident shows that annealed graphene end results can be changed with different temperature and time applied. The annealing process helps minimize FLG flake-to-substrate defects, improves print resistance, and also cleans any polymer contaminants on the graphene surface.

Multiple conductivity measurements were applied uses a multimeter. Resistances of all 5 printed lines were measured cross the gap for every 1 mm. Previous study given results show that laying layers of wet ball milled graphene prints on a substrate contains a sheet resistance of 133 Ω/□, however, by applying thermal annealing to the same sample in a standard oven at 280 °C can help reduce the sheet resistance to as low as 36.75 Ω/□.

2.4.4. Biocompatibility Testing with N27s

3 mL RPMI media was first added into each well plate and a 50 µL cell sample was added
into the media. N27 cells were observed under inverted microscopy after incubating between 24 hours and 96 hours. Figure 2-21 indicts the growth rate of the cells, demonstrating that after 96 hours, the cells accounted for 80% of the live screen area. A cell live-dead assay was performed to confirm the survival rate of cells grown on graphene.(75,76) It was expected that cells would grow on top of the annealed Kapton and graphene, since polyimide materials are often used for biosensors because of their strength and broad compatibility with many devices.(77) N27 cells were fully expanded across the gap, connecting both sides of the graphene prints (Figure 2-19).(78,79)
Figure 2-18. Cells checked after 48 hours

Figure 2-19. Cells checked after 96 hours
Figure 2-20. Inverted microscope image of N27 cells (Control)

Figure 2-21. Shows the results of cells live-dead assays after 72 hours of incubation
Figure 2-22. Cells Live-Dead Assays after 72 hours of incubation show s cells survived on graphene prints

From cell live-dead assay results, N27 cells were successfully grown on the graphene and Kapton substrate, which proving our earlier hypothesis.(79,80) Based on counting green staining marks, the 90% of the cells survivability has been calculated in Figure 2-23. Graphene prints showed no harmful effects to cell growth, from counting the green and red stains this again proves the inkjet printing graphene method of making biosensors can be further applied and applied in the future.
Figure 2-23. Percentage of Live Cells

There have been discussion in the tissue-engineering literature about interaction of graphene with biomolecular complexes that interfered with its biological safety and toxicity. Many reports have claimed that oxidative stress is one of the mechanisms involved in cytotoxicity due to carbon based nanomaterials, with a possible mechanism of toxicity graphene’s sharp edges that might cause cell membrane damage by physical interaction. Studies have also found evidence that graphene oxide is less toxic than graphene, while reduced graphene oxide and hydrogenated-graphene, and large flakes and more aggregating graphene solutions are much more toxic to neural cells. Such evidence support our setup described above, in which there was no detectable cytotoxicity to the cells, with optical images showing that only a small proportion of cells were affected at the locations studied. This result could be beneficial for future N27 cell electrophysiology studies.
2.5. Conclusion

In conclusion, an easily applied and inexpensive biosensor design has been presented. Based on cell live-dead assays, this design can maximize printed FLG conductivity after annealing process, it also proved beneficial for use with biocompatible cells, with respect to both neuronal cell adhesion and neuronal cell proliferation. This design has proven that pristine graphene after thermal treatment does not produce harmful effects on neuronal cells. In addition, graphene printed on Kapton Polyimide exhibited no detectable adverse effects on cell multiplication, mitochondrial morphology and cell stress.(18) This highlights the bright promise of graphene in future studies, representing long-term and stable biomedical applications, especially in bioelectrical studies on N27 cell electrophysiology.
CHAPTER 3. CONCLUSION & FUTURE WORK

In conclusion, from the results discussed and presented in Chapter 2, the few-layers graphene made by BSA, applied mechanical exfoliation method, using wet ball milling and kitchen blender techniques have excellent properties in biochemical and biomedical applications. The graphene prints on Kapton Polyimide using this custom printer setup showed an excellent flexibility, along with good conductivity and decent water resistances. Based on cell live-dead assays, the biocompatibility of our biosensor design has been proved for neuronal cell use, both on cell adhesion and cell proliferation. It also proves that pristine graphene does not have harmful effects on neuronal cells. In addition, graphene printed on Kapton Polyimide shows no detectable adverse effect on the cell multiplication, mitochondrial morphology and cell stress. Moreover, this sensor setup has an easy application and inexpensive features compared to other sensors that have been studied in other researches.

For the future work, a more practical printing method will be needed for this research. The custom printer design that was used in this study has several major problems: firstly, the CNC G-code could not be efficiently utilized during the printing process, which made the process take a long period of time. This issue will cause inaccuracy and less efficiency during printing process. From experiences operating the printer, the flatness of the substrate is very necessary for making consistent prints. Right now, to improve the prints quality, we could modify the printer with higher accuracy motors; calibrating the CNC software and adjusting CNC tracks to make the machine operate smoothly. Second, the graphene solvent must be mixed with a proper ratio for smooth and flexible printing results. In the future study, a stable way of mixing graphene solvent before printing process is crucial because if the solvent is too dense the needle would most likely be jammed by unbroken graphite particles, but if the
solvent is diluted, it may take many passes on one line to form a better, visible pattern. Therefore, finding the perfect mixing ratio is also important to the future printing process.

As this study continues, the next step will be focusing on electronic signals that are caused by damaging the N27 cells. This study requires high accuracy microelectron equipment from other labs. For the future cell electrophysiology study, the challenge continues to exist. To find the correct signal that N27 cells generate, noise cancelation methods must be applied. For the future, ideas could be found from doing more literature reviews that relate to the cell bioelectrical signals recording. One research used electrodeposited PtNps and PTE ink for the bioelectrode, using a potentiostat for the electroanalytical measurements. Another literature states that using graphene oxide (GO) served as specific affinity probes with LDI substrate for target cells analytes. Looking for an application that is the most efficient and accurate method would be a major challenge for the future study. For the future, our group will exceed the major goal of this research: capture the most accurate N27 cell electrophysiology signal using this biosensor design.
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