Design and rapid prototyping of printed graphene electrochemical biosensors for sensitive monitoring of pesticide levels for agricultural use

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Design and rapid prototyping of printed graphene electrochemical biosensors for sensitive monitoring of pesticide levels for agricultural use

by

John Avert Hondred

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Mechanical Engineering

Program of Study Committee:
Jonathan Claassen, Major Professor
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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 dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2019

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ABSTRACT

While the use of pesticides (herbicides and insecticides) are critically important to meet the current and future food demands (increases crop yield by up to 40%), their overuse has shown long-term detrimental impacts on the environment from polluting watersheds used for drinking water to eutrophic “dead zones”. Current pesticide soil measurement methods (chromatography) are costly, require trained technicians, and take days to analyze; thus, farmers are taking an “over-application approach” which is pollution the environment and waterways. A disposable pesticide soil sensor would provide farmers the opportunity of precisely regulating the application of pesticides in an independent and economical fashion. Electrochemical biosensors provide a unique ability to quickly detect analytes with a handheld device and low-cost sensors; however, the detection limit and sensitivity of these biosensors are inadequate for current applications.

This dissertation addresses this issue with the following focus in mind: 1) Increasing the enzymatic efficiency of organophosphate hydrolase by strategically functionalizing to nanomaterials [e.g., 17-fold increase in Vmax when functionalized to gold nanoparticles vs free enzyme]. 2) Develop a low-cost, rapid, and high-resolution manufacturing method to pattern solution-phase graphene [i.e., inkjet maskless lithography (IML), line resolution ~20 µm, sheet resistance ~50 Ω/sq]. 3) Enhance the electroactive surface area by nano/microstructuring the graphene surface [3D petal-like graphene morphology] using laser annealing. 4) Increase the electrochemical surface area by incorporating macro and micro pores [2.2 times higher sensitivity with the inclusion of macropores] in the graphene surface.

This work demonstrates the manufacturing of simple, low-cost electrochemical biosensors which is suitable for rapid in-field detection of organophosphates. The fabricated
Graphene biosensors demonstrate high sensitivity, large linear sensing range, and ultra-low detection limits. Additionally, while this work is tailored towards a disposable pesticide sensor, the manufacturing techniques, sensor designs, and biosensor principle are a platform technology that could be amenable to other applications such as healthcare screening, drinking water monitoring, and even bioterror agent detection.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized into multiple chapters, each with a manuscript that has been published or is being prepared for submission into peer reviewed scholarly journals.

Chapter One

Chapter one is a general introduction to the advantages and disadvantages of using pesticides for agricultural use and their potential toxicity. This chapter provides background for the monitoring of pesticides and the motivation associated with the use of electrochemical biosensors.

Chapter Two

This chapter provides a literature review of the design and manufacture of printed graphene electrochemical biosensors. It provides a brief overview of the principle/mechanism of operation and gives the reader an introduction into the design of electrochemical biosensors, specifically on flexible, low-cost substrates. This review article depicts a variety of manufacturing printing processes for patterning solution-phase graphene, techniques for post-processing and methods for modifying and enhancing the electroactivity of the transduction surface. Finally, this chapter concludes with current applications of carbon based flexible electrochemical biosensors, challenges in the field and opportunity for future work.

Chapter Three

This chapter covers the work of increasing the enzymatic activity of phosphotriesterase (PTE$_3$), a type of organophosphate hydrolase, by strategically functionalizing the enzyme to various size gold nanoparticles (5, 10, 20 nm). This chapter demonstrates that nanoparticle morphology plays a key role in impacting enzyme activity. The small diameter of nanoparticles
and their high curvature allows for increase center-to-center distance between adjacent enzyme while limiting unfavorable protein-to-protein interaction which enhances the catalytic abilities of the enzyme and increases diffusion rates of substrate to and from the enzyme. Using various size gold nanoparticles (AuNP), the enzyme kinetics of PTE$_3$ are enhanced ($V_{\text{max}}$ increased 17-fold when functionalized to 20 nm AuNP) by ratiometrically immobilizing on the surface through histidine tag.

**Chapter Four**

This work develops a new manufacturing method (coined inkjet maskless lithography (IML)) to rapidly manufacture graphene electrodes with high resolution (~20 µm line width), even on flexible heat-sensitive substrates. This method utilizes solution-phase graphene, a low-cost alternative to carbon vapor deposition (CVD) graphene. Using a material inkjet printer a polymer ink is printed which acts as a sacrificial layer to pattern solution-phase graphene. This method presents a fast, economical printing process without the need for cleanroom technology. Through this method, high resolution patterns can be fabricated in complex geometries such as interdigitated electrodes (IDEs) which decreases electroactive impedance making a more sensitive electrochemical sensor. IML graphene electrodes demonstrated a high degree of electroactivity and conductivity making them an excellent transduction material for electrochemical sensing demonstrated through the development of a sensitive hydrogen peroxide amperometric sensor and a potassium chloride impedance IDE sensor.

**Chapter Five**

Chapter five uses the IML technique method to design and manufacture a sensitive electrochemical biosensor for organophosphate detection using PTE$_3$. Moreover, this chapter describes the use of laser processing to anneal IML printed graphene, which alters the graphene
flake orientation by tuning graphene flakes vertically which increases the electroactive surface area making a more sensitive electrode. This chapter demonstrates that the IML graphene electrode utilizing PTE is an effective biosensor for detection of paraoxon (a model organophosphate) even in real world samples (tap water, river water, and filter soil slurry). Hence this chapter presents a scalable printing process for fabricating organophosphate pesticide biosensors that are suitable for in-field applications.

Chapter Six

Chapter six improves on the IML process by increasing the electroactive surface area of patterned solution-phase graphene by increasing the porosity and graphene edge plane density through the construction of a multidimensional architecture *via* salt impregnated inkjet maskless lithography (SIIML) and CO$_2$ laser annealing. Using salt crystals as porogens, macrosized pores (~25 µm) are patterned directly into the graphene ink. Subsequently, microsized pores (~100 nm to 2 µm in width) with edge plane defects are etched in the graphene lattice structure by laser annealing with a CO$_2$ laser, which enhanced the electroactive nature of patterned graphene. This chapter demonstrates that CO$_2$ laser annealed and SIIML graphene electrodes can improve the electrochemical device performance by an organophosphate biosensor using the enzyme acetylcholinesterase. Additionally, as SIIML graphene serves as a platform technology a high energy density of 0.25 mW h cm$^{-3}$ at a power density of 0.3 W cm$^{-3}$ supercapacitor was constructed. The supercapacitor was able to power an LED and as it was constructed on flexible substrate could be integrated into wearable technology.


Chapter Seven

The last chapter in the dissertation, chapter seven, provides conclusions from the previous work. It analyses shortcomings in the current biosensor technology field and suggests areas that should be further researched. Finally, it recommends how the work discussed in this dissertation should be continued.

Introduction

Motivation

Currently, there are over 7 billion people in the world and the population is predicted to increase by roughly 30 percent by 2050 and double in the next century.\(^1\) To feed this increase in population, current crop yield will need to increase by an estimated 70% its current production rate.\(^2\) Through the use of technological advances (e.g. genetic engineering, and improved farming equipment and practices) and the adoption of management practices, crop yield has been increasing at roughly the same rate to account for the rise in population. Central to the agricultural advances are the development of pesticides (herbicides, insecticides, fungicides, and nematicides) which prevent weeds, insects, worms, and the like from damaging crop yield. The use of these chemicals account for roughly 30-50 percent retention of yield and are crucial to feeding the rise in global population.\(^3\)

Approximately 1-2.5 million tons of active pesticide ingredients are applied to the environmental each year, making them the second most environmentally applied chemical behind fertilizers.\(^4\)-\(^5\) Despite their significant contribution in agricultural production, pesticides are toxic to humans and the environment. Due to their over application, pesticides such as organophosphates are leaching from the soil and water run-off from the fields are migrating into the rivers and precious water reservoirs used for drinking water. Pesticides have even been
found in measurable quantities in soil,\textsuperscript{6} living organisms (e.g., stream macroinvertebrates),\textsuperscript{7} surface and groundwater,\textsuperscript{8-9} and rainwater run-off.\textsuperscript{10} Even at low concentration, pesticides have far reaching and diverse negative effects from potential links to autism spectrum disorder\textsuperscript{11} to honeybee colony decline.\textsuperscript{12} Organophosphates (a type of insecticide) in particular are extremely toxic to humans and can cause comas, impotence, and even death.\textsuperscript{13} Even more severe, many of these pesticides can be synthesize into G-type chemical warfare weapons such as Soman and Sarin, which were used in the 1999 Tokyo subway attack poisoning over 600 victims.\textsuperscript{14-15}

The U.S. Department of Agriculture (USDA) currently uses gas/liquid chromatography coupled with mass spectroscopy to detect and monitor the presence of pesticides (e.g., organophosphates, organochlorines, carbamates, triazines, triazoles, pyrethroids, neonicotinoids) in approximately 85 agricultural commodities\textsuperscript{16} and in more than 10,000 samples a year through the USDA-Pesticide Data Program (PDA).\textsuperscript{17} While these detection techniques have been demonstrated to accurately, sensitively, and reliably detect and quantify pesticide concentrations in water samples, they require laboratory processing. Samples need to be taken, shipped to a facility and trained technicians run extensive assays. This process is time consuming (minimum of 2-3 days) and expensive; requiring sample preparation, specialized personnel and instrumentation, and often require harmful solvents and chemicals. Furthermore, these methods are unable to provide real-time, in-field feedback of pesticide levels from soil sample making them inadequate for screening pesticide overapplication or water contamination. Moreover, experience laboratory equipment such as chromatography are limited or non-existent in low economic and third world countries.
Advantages of Electrochemical Biosensors

Electrochemical biosensors present an attractive alternative to high-cost and time-consuming chromatography or mass spectroscopy. They can be equipped with hand-held potentiostat devices that present facile and portable operations. Moreover, they can provide rapid results, require minimal volume with high accuracy, and are sensitivity with low detection limits. Biosensors are typically highly selective due to the specific affinity of the biological recognition element and interacting substrate.18 Typical recognition elements include: enzymes, nucleic acids, aptamers and antibodies. Enzymes are the most commonly used biological recognition agent,19 but often lack sensitivity and suffer from high detection limits not suitable for real-world application.

Nanotechnology has emerged in the last decade as an exciting new field, especially for analytical chemistry.20 Due to their small size, nanoparticles hold intrinsic characteristics that exhibit unique chemical, physical and electrical properties. Specifically, nanoparticles have high surface to volume ratios which can dramatically improve electrochemical sensor and biosensor performance.21 This unique feature provides excellent scaffolds for functionalizing biological recognition elements, enzymes in particular. Medintz’s group has demonstrate in multiple different manuscripts22-24 that the assembly of enzymes on nanoparticle accelerates the enzymatic activity. Multiple different factors such as size, shape, surface chemistry, curvature, enzyme ratios and complex orientation can affect the enhancement and activity of enzyme-nanoparticle complex.25 Additionally, nanoparticles help to “electrically wire” enzymes to the electrode, enhancing the electron transfer between the redox center of the protein and the electrode surface and improves catalysis which increases the electrochemical reaction.26-27 Specifically, carbon-based nanoparticles provide unique physiochemical
platforms for enzyme based electrochemical biosensors. Graphene in particular has shown superior performance in terms of electroactivity\textsuperscript{28-30} and can directly fabricated into flexible, low-cost sensors.

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CHAPTER 2. THE DESIGN, MANUFACTURE, AND APPLICATIONS OF PRINTED NANO-CARBON ALLOTROPE FLEXIBLE ELECTROCHEMICAL BIOSENSORS

A paper prepared for submission to ACS Nano

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keywords: Graphene, wearables, RFID, origami, disposable, test strip, flexible

Abstract

Nano-carbon allotropes provides an attractive electrochemical platform material for flexible electrochemical biosensors due to their high electrochemical surface area, biocompatible surface, fast heterogenous charge transport, flexibility and high conductivity. Moreover, carbon nanomaterial inks can be patterned with current printing technology and integrated into roll-to-roll manufacturing which significantly lowers cost per device, often the limiting factor for disposable sensors. In particular graphene due to its two-dimensional nature, synthesized through chemical/mechanical exfoliation from low cost graphite, can by fabricated into conductive inks that can be printed into economical flexible electrochemical biosensors with exceptional sensitivity. Additionally, post processing (annealing) and surface modification (nano/microstructuring, porosity, doping) of the printed graphene can improve the conductivity, electroactivity, surface area, and robustness of this transduction layer while also providing a unique biocompatible platform for biorecognition immobilization. Printed carbon-based flexible electrochemical biosensors have garnered substantial interest in numerous different fields including food safety, biochemical weapon screening, environmental
pollution detection, personal health monitoring and diagnostics, medical screening, and agricultural.

**Introduction**

The burgeoning field of flexible electrochemical biosensors has grown to a multi-billion-dollar market due to their unique and beneficial characteristics. These biosensors present useful devices for low-cost point-of-care screening, infield disposable test sensors, wearable monitoring. Flexible electrochemical biosensors provide attractive features due to their inherent flexibility that allows them to conform to the inside of packaging for space saving or non-planar shapes, adhere directly to a surface such as skin or food, or attach to an interface system such as a microfluidic/flow-cell or lab-on-chip. Flexible electrochemical biosensors are revolutionizing the point-of-care and direct infield sensing in a variety of fields, such as: food safety, environmental pollution detection, health diagnostics and personal health monitoring.

Printed carbon-based electrodes present an attractive benefit for flexible electronics as they provide an economical alternative compared to high cost wafer technology or metal deposition. Nano-carbon allotropes (e.g. carbon dots, carbon nanotubes, graphene) provides electrochemical benefits such as high surface area, fast heterogenous charge transport, high conductivity, reduced electrode fouling and a biocompatible surface for biological recognition functionalization. Carbon nanomaterials can also be fabricated into conductive inks and utilized in low cost printing processes (e.g. screen printing, inkjet, gravure). Low-cost and lightweight carbon-based biosensors can be fabricated in mass production through the use of flexible substrates (synthetic polymers, paper, textile) while incorporating rapid and economical printing methods (e.g. roll-to-roll manufacturing), which dramatically decreasing
the cost per device, typically the limiting component of new emerging technology. Due to their low-cost, flexible carbon biosensors are emerging as a top competitor for disposable sensors. Moreover, carbon printed electrodes can be disposed of in high quantities without damage to the environment, unlike metal printed electrodes which can cause heavy metal pollution. Carbon based flexible electrochemical biosensors have been developed for multiple different applications including: wearables for physiological monitoring, origami paper sensors, RFID biosensors, and disposable low-cost test strips.

Graphene, in particular, has shown great promise in the area of flexible electronics and electrochemical sensors due to its 2D nature which can be printed into well-defined patterns on a variety of substrates with current printing technology. Graphene has exceptional mechanical properties (fracture strain of 25% and a Young’s modulus of > 0.5 TPa) that provide it flexibility and in some cases even stretchability for printed electronics. Moreover, graphene has excellent conductivity and high surface area which increases the electrochemical activity by enhancing the electrolyte/electrode interface area improving sensitivity. Graphene can also be easily modified (e.g. doping, microstructuring, electrodeposition of nanoparticles, chemical modifications) to increase electrochemical activity, functionality, or hydrophobicity.

This review focuses on the recent progress of the design and fabrication of nano-carbon allotropic based flexible electrochemical devices with a focus of the use of graphene. It is split into four different sections (design, manufacture, applications, challenges/future work): First, we describe the basic design and mechanism of operation for flexible nano-carbon allotropic electrochemical biosensors, including the flexible substrate material, carbon transduction materials, and biorecognition agent. Second, we summarize the different fabrication technique
used to printed graphene materials on flexible substrates, post-processes, surface modifications and immobilization strategies (specifically for carbon-based materials). Third, we highlight several different current applications of flexible printed carbon-based electrochemical biosensors such as wearables, wireless transmitted (RFID), origami and disposable biosensors. Finally, we discuss current challenges and opportunities that lie ahead.

**Design**

**Basic Electrochemical Operations**

The International Union of Pure and Applied Chemistry (IUPAC) have defined an electrochemical biosensor as “a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element.”

Simply speaking, it is a device that is able to detect an analyte of interest using a biological agent and converts that response into an electrical signal to be interpreted by a user. Flexible electrochemical biosensors operate similar to “rigid” electrochemical biosensors; however, they are designed and fabricated on a flexible substrate allowing the device to bend, twist, conform, and in some cases even stretch for different applications.

Flexible electrochemical biosensors are composed of three different main components and illustrate in Figure 2.1a: 1) A flexible substrate that provides mechanical support to the system. 2) A conductive and electroactive transduction material that serves as an electrode for electrochemical process and biological scaffolding. 3) A biorecognition agent that is specific to the analyte of interest. Typically, electrochemical biosensor operates by six essential steps as depicted by Figure 2.1b. First, an analyte of interest diffuses to the electrode surface. Then,
a biological recognition agent (e.g. enzyme, aptamer, antibody, nucleic acids, bacteria, etc…) reacts with the target analyte by catalysis, binding, or altering its structure. The electroactive surface electrode (transduction element) then monitors this change in biological detection through an electrochemical process (e.g. oxidation, reduction, change in impedance/resistance or potential). Finally, a potentiostat monitors and detects this electrochemical process and displays it for the end user to interpret.

Figure 2.1 Electrochemical biosensor design a) schematic of a flexible electrochemical biosensor, b) six essential steps of a biosensor.

**Flexible Substrates**

The substrate of the electrochemical biosensor provides support to the device, holds all components together and provides stable operating conditions; it is the main backbone or building block of the biosensor. The mechanical properties of the substrate (mainly flexibility or stretchability) play a crucial role in defining the physical dynamics and application of the biosensor. However, other physiochemical properties such as thermal/chemical stability, solvent resistance, moisture/gas resistance, biocompatibility, and biodegradability govern manufacturing of the flexible electronics and environmental stability of the electrochemical biosensor. For instance, a material that is highly flexible and even stretchable may contour a nonplanar, flexing surface such as the human body; but in a different situation, a more robust
sensor such as inside of a food package may be required. There are three main different categories of flexible substrate: synthetic polymers, paper, and textiles.

**Synthetic Polymers**

Synthetic polymers (plastics) are currently the most popular substrate for flexible electrochemical biosensors, due to the high degree of versatility between physical, chemical and mechanical performance. Unlike many other substrates, polymer films do not absorb liquids and can be engineered to be thin and bendable while retaining a smooth surface. Polymer substrates are divided into two different groups: semi-crystalline and amorphous.

Semi-crystalline polymers tend to have a highly ordered molecular structure which give them a sharp, higher melting temperature (they do not soften gradually with increasing temperature). Semi-crystalline polymers used in flexible electrochemical biosensors include polyethylene terephthalate (PET), polyethylene naphthalate (PEN), and polyether ether ketone (PEEK). PET is a polyester material that is often used in clothing or plastic containers. It is an excellent electric insulator, thermally stable, low-cost, and is chemically inert. Additionally, it can be easily manufactured into different shapes and sizes as it can be pressed into thin film or spun to fibers (wearable materials). For example, Lanlan et. al. designed a multiplexing biosensor using a custom-made inkjet printed carbon electrode on PET which was flexible and bendable (See Figure 2.2a). The entirely inkjet printed amperometric multiplex biosensor was able to detect glucose, lactate, and triglycerides in real time with good sensitivity. PEN is also a polyester with more chemical and thermal stability and higher hydrolytic resistance than PET; however, it is more rigid. PEEK has excellent mechanical and chemical stability even at high temperatures. Krishna et. al. fabricated a sub-millimolar glucose biosensor on PEEK
using a glassy carbon ink with a graphene and nickel nanoparticle modified working electrode.\textsuperscript{33}

Amorphous polymers have random molecular structure and are typically more sensitive to stress failure.\textsuperscript{34} Amorphous synthetic polymers used in flexible electrochemical biosensors include polycarbonate (PC), polymethyl methacrylate (PMMA), and polyimide (PI). PC is a durable material with high impact resistance but low wear resistance; it has been used in electrical applications due to its good electrical insulation and high dielectric constant. Wang et. al developed a strong bonding microwave irradiation welding method for improving the adhesion of MWCNTs on PC which demonstrated excellent flexibility.\textsuperscript{35} PMMA (also known as acrylic) is a cheaper alternative to PC but has lower temperature resistance and is more brittle than many other synthetic polymers. Many researchers have used PMMA as a substrate for graphene transfer.\textsuperscript{36-37} PIs (commercially available as Kapton films) are one of the most popular industrialized flexible substrates. It exhibits excellent thermal, chemical, and mechanical stability.\textsuperscript{38} They can be commercially synthesized with multiple different properties by slight modification to their molecular structure. PIs exhibit high tensile strength and low creep even at elevated temperature (~350°C).\textsuperscript{39} They have been used extensively in commercial/industrial applications for lightweight, flexible electronics. We have shown that inkjet printed graphene on polyimide film can have low sheet resistance (~700 Ω sq\textsuperscript{-1}) and is mechanically flexible, with no increase in resistance with over 100 bending cycles, Figure 2.2b.\textsuperscript{23}

**Paper**

Paper is an inexpensive and attractive substrate for flexible electronics. It is a semi-synthetic/semi-natural substrate. Due to its extremely low-cost, paper-based electrochemical
biosensors are in increasing demand in resource limited countries for use as disposable electrochemical biosensors.\textsuperscript{40} Paper-based substrates are porous materials that absorb liquid and are often used in conjunction with lateral flow systems.\textsuperscript{41} There are a variety of different paper types which can be tailored with different physical and/or chemical characteristics. For printed electronics, glossy paper and filter paper are often used which are composed of cellulose fibers blended with an inorganic filler. Paper substrates are prone to tearing and have stability problems in wet environments which dramatically limit their use.\textsuperscript{17}

One of the greatest advantages of flexible paper base sensors, is that the paper substrate not only provides mechanical support of the transduction electrodes, but it also can be used as flow cell to wick solutions to the electrode interface.\textsuperscript{41} The first paper based electrochemical biosensor that used paper in this fashion was published by Henry’s group which simultaneously detected glucose, lactate, and uric acid with a screen-printed carbon electrode, Figure 2.2c.\textsuperscript{42} We have also demonstrated the use of inkjet printed graphene on nanocellulose\textsuperscript{43-44} and cellulose-based paper with low resistance and good flexibility even after 100 washing cycles, Figure 2.2d.\textsuperscript{23}

**Textiles**

Textile are a type of flexible substrate that can be fabricated through fibers, filament, yarn, or threads and are typically a cotton, silk, wool or synthetic blend (nylon, polyester). The textile can be knitted, woven, or nonwoven (matted). They provide unique physical and chemical properties that are especially suited for wearable on-body biosensors where flexibility, lightweight, durability, and even stretchability are paramount. Similar to paper substrates, textiles are porous materials that often absorb liquids; however, they provide stronger mechanical strength and are much more resilient to tearing. Textiles substrates present
a useful advantage for monitoring under the “wear-and-forget” paradigm, as the electrochemical biosensor can be integrated directly into a user’s garment. Further information on patterning flexible electrochemical circuit on textiles for wearable application can obtain from the following book chapter.

While not a biosensor (no biological molecule used), a Korean group developed an electrochemical gas fiber sensor that can be directly integrated into clothes, Figure 2.2e. This electronic textile (e-textile) gas sensor is composed by electrostatically adhering graphene to cotton or polyester yarn and can be embroidered into commercial textile fabric (such as a lab coat). When connected to a custom-made small electronic board, an LED illuminates when exposed to certain gas concentrations. The sensor showed selectively to NO\textsubscript{2} gas down to 250 ppb. Furthermore, the sensor showed reliability under several washing treatments and over 1000 bending cycles (1 mm radius).

![Figure 2.2 Flexible electrochemical biosensors on different types of substrates](image)

Figure 2.2 Flexible electrochemical biosensors on different types of substrates a) Inkjet printed multiplex (glucose, lactate, and triglyceride) biosensor on semi-crystalline PET substrate.
Transduction Materials

Carbon Paste (Graphite, Glassy Carbon, Amorphous Carbon)

Since its introduction, by Norman Adams in 1958, carbon paste has become one the most widely used electrode materials for its excellent physiochemical and electrochemical properties. Carbon-based electrodes have been commonly used due to their low-cost, fast heterogeneous charge transfer, conductivity and biocompatibility. Carbon paste is formed from a mixture of graphitic powder (typically 5-20 µm flake size) and liquid paste binder, however, some mixtures include soot/charcoal, acetylene black and glassy-carbon. Despite the typical use of insulating binders (paraffin or silicone oil), carbon paste electrodes exhibit very low resistance, on the order or tens of ohms. One of the main benefits of carbon paste electrode is their ease of renewal via mechanically removing the top surface of the electrode through polishing. The electroanalytical properties of carbon paste electrodes can be easily modified during fabrication, but they typically have a wide potential window, about -1.0 to +1.0 V (which fluctuates based on pH and solution concentration). However, anodic potentials as high as +1.85 V vs. SCE and cathodic potentials as low as -2.0 V vs. Ag/AgCl have been achieved. For a more detailed analysis over the history and physiochemical properties of carbon paste electrodes, the reader is directed to the following review article. Carbon paste electrodes heavily depend on their binding material to provide mechanical stability but due to
their thick film deposition methods have poor flexibility (tend to crack when flexed).\textsuperscript{53} However, some researchers have increased their flexibility through use of cellulose acetate matrix,\textsuperscript{54} or wax flexible modifier loading.\textsuperscript{55}

**Carbon Nanotubes**

Carbon nanotubes (CNTs), whether single walled (SWCTs) or multiwalled (MWCNTs) are hollow cylindrical tubes made up of entirely carbon atoms (sp\textsuperscript{2} hybridized hexagonal honeycomb structure) with extremely high aspect rations (length/diameter). MWCNTs are multiple concentric tubes encircling one another, while SWCNTs are composed of a single cylinder. CNTs possess many of the same properties of carbon paste electrodes but due to their 1D structure, they provide enhanced electronic properties, higher electrochemically active surface area and increased diffusion rates; all of which increase the electrochemical sensitivity, lower detection limits, and improve transfer kinetics over standard carbon paste electrodes.\textsuperscript{56-58}

Carbon nanotubes have been synthesized by many different techniques; however, the three main processes are: arc discharge, laser ablation/vaporization, and carbon vapor deposition (CVD).\textsuperscript{59} Arc discharge forms CNTs on a graphite electrode by applying a current through two electrodes.\textsuperscript{60} Laser ablation uses a laser to vaporize graphite in a high temperature tube which forms MWCNTs or SWCNTs if metal nanoparticle precursors are used.\textsuperscript{61} CVD uses a high temperature furnace which grows CNTs on metal catalyst by flowing hydrocarbon gases over the surface.\textsuperscript{62} CVD is most widely used for commercial applications as it is able to grow aligned CNTs as a “forest”.\textsuperscript{63} Regardless of the method, CNT fabrication requires expensive fabrication and even through rigorous purification, it is difficult to remove all metal
impurities. A more detailed analysis over CNTs synthesis can be found at the following review article.64

Due to their high cost, CNTs are typically used to modify the electrode surface and not often used as the entire transduction layer, although some research such as Costa et. al. has inkjet printed entirely CNTs transduction electrochemical biosensors.65 CNTs can cost over a thousand dollars per gram (for high purity single walled CNT) due to the high manufacturing and purifying cost associated with fabricated them.66 Therefore, CNTs are typically incorporated into/onto the carbon electrode by direct growth or adsorption on the electrode surface, or incorporating them in a carbon paste ink.57

**Graphene**

Graphene, a single atomic thick 2-dimension carbon lattice (sp² hybridized structure), has attracted a lot of scientific and technological attention since its discovery in 2004, due to its unique physiochemical properties of excellent conductivity, high mechanical strength, flexibility/stretchability, high surface area, efficient electrochemical activity, and ease of functionalization.67-69 While graphene has many of the same attributes of other carbon materials, it distinguishes itself through many unique characteristics, for example: 1) it is a semiconductor with zero-order band gap, classifying it as a semi-metal, 2) exhibits high carrier mobility (up to ~10 000 cm² V⁻¹S⁻¹), 3) large theoretical surface area (2630 m² g⁻¹), and 4) high electrical conductivity (64 mS cm⁻¹).70-72 Moreover, graphene has extraordinary electrochemical properties such as large potential window, fast heterogenous charge transport, and low charge-transfer resistance.73-74

Graphene can be synthesized by two different approaches “bottom-up” and “top-down”. Bottom-up method consists of growing individual graphene sheets through epitaxial
growth on SiC, CVD, or synthesis through organic precursors. Bottom-up methods are much more expensive but are able to produce defect-free/pristine, large-area graphene sheets (diameter over 18 inches). On the other hand, top-down methods are simpler and cheaper as they use bulk graphite and attempt to exfoliate and break the strong Van der Waals energy (5.9 kJ mol\(^{-1}\) carbon) holding the individual graphene sheets together. Typically for mass-production and high-volumes, solution-chemistry based top-down methods are employed, especially for fabrication of electrochemical biosensors. These top-down methods oxidize bulk graphite (typically a modified version of Hummer’s method), mechanically exfoliate the graphene oxide flakes, and then reduce the graphene oxide to reduced graphene oxide (often referred to as just graphene). Top-down method typically result in very low yield and small flake size, so filtration steps such as ultra-centrifugations are employed to isolate and concentrate the single layer graphene. The solution synthesized graphene can be easily incorporated into a graphene ink and be patterned with conventional printing techniques.

**Manufacture**

**Printing Processes**

The manufacturing/patterning method used to fabricate flexible electrochemical devices significantly impact their simplicity, resolution, application, and cost. Lithography techniques using wafers such as photolithography, e-beam, or ion-beam lithography produce high resolution (nanometer feature size) patterns for high performance devices. However, these clean-room processes come at a high financial cost which drives the price per device up, many times setting the cost of the device above market penetration (e.g. medical strips, RFID biosensors, disposable agricultural sensors). Additionally, these processes often require transfer from hard/rigid substrates that are thermally stable such as silicon wafers or glass.
Printing technology has emerged in the last decade in response to the high production costs and need flexible electronics. Inexpensive printing processes can produce large quantities of devices on flexible substrates while maintaining precision and accuracy. Using flexible substrates and printing processes allow for fabrication on large areas that would not be possible with commercial wafer patterning technology. There are numerous printing approaches that can pattern different novel ink formulation or chemically alter the substrate/laminate material (reader is directed towards review article by Dahiya); however, our review will focus on printing processes that can pattern carbon materials (graphene in particular) for flexible electronics such as: screen printing, flexography/gravure, inkjet printing, 3D printing, aerosol and laser scribed.

**Screen Printing**

Screen printing is the most popular and mature electronic printing process for flexible electronics as it has been implemented in the electronic community to print interconnects on printed circuit boards. Screen printing is a template-based method that uses a rubber squeegee to force a thixotropic fluid ink through a wire mesh/stencil and onto a substrates. When forced through the stencil/mesh the viscous ink undergoes shear thinning facilitating the penetration though the template and upon contact the substrate returns to its viscous state.

Screen printing of carbon materials, especially pristine graphene, is often more difficult than many metallic inks, as forming a viscous high carbon content inks tends to aggregate forming poor resolution or blocking the stencil. In order to increase the viscosity of the carbon ink and modify its surface tension, polymer binders are usually added which often have unwanted and negative effects on the patterned carbon (e.g. lowers conductivity, changes in dielectric constant). Therefore, much care must be taken into designing a carbon ink that has
high carbon content (to form conductive patterns) with the right viscosity but also remain a colloidal well dispersed mixture. Frisbee’s group designed a highly concentrated (~80 mg mL\(^{-1}\)) graphene ink using an ethyl cellulose polymer binder and ethanol as a solvent.\(^{80}\) Using this ink, they were able to pattern graphene lines down to 5 \(\mu\)m with a custom-made silicon stencil, Figure 2.3a. The screen-printed graphene lines were patterned on polyimide films and exhibited excellent mechanical flexibility and high electrical conductivity (~18 600 Sm\(^{-1}\)).

**Gravure/Flexography**

Gravure printing is a contact printing method that uses an engraved cylinder that roll over top of a flexible substrate and coats it with ink in a desired pattern. A cylinder is manufactured using electromechanical means or etched with a laser, then electroplated with chrome to prevent wear.\(^{81}\) During printing, the cylinder is coated with the desired ink from a reservoir underneath or nozzles dispensing ink from above. The cylinder is then doctor bladed to remove extra ink from the rotating cylinder and contacts the substrate to deposit the ink in the desired pattern from the etched wells.\(^{82}\) Flexography transfers a pattern in a very similar fashion, except instead of using an engraved cell, it uses a raise pattern (similar to a stamp).

The viscosity and surface tension of inks used for gravure or flexography printing must be compatible with the cell width/depth ratio used on the cylinder. Low viscosity inks are often used as they allow for increased processing speed and improve line resolution by emptying the grooves faster.\(^{83}\) Hersam and coworkers developed an ethyl cellulose graphene ink using terpineol (10% by weight) that could be patterned by gravure printing with line resolution down to 30 \(\mu\)m, Figure 2.3b.\(^{84}\) The patterned graphene lines demonstrated excellent flexibility and conductivity as high as 10 000 Sm\(^{-1}\).
**Inkjet Printing**

Inkjet printing is a drop-on-demand printing technology and is arguable the most versatile of all solution-based printing processes, Figure 2.3c. Inkjet printing does not depend on fix stencils, cylinders, or masks. As inkjet printing deposits droplets based on a pixelated pattern, new designs can be quickly uploaded and printed, making inkjet printing an excellent rapid prototyping technique.

Colloidal suspensions or inks are jetted through micrometer sized holes in a nozzle head. A variety of different mechanisms exist to provide jetting potential; however, the two most popular are thermal and piezoelectric. Thermal inkjet uses small thermal resistors; when a voltage is applied, the resistor quickly heats up and vaporize a portion of ink creates an air bubble that causes a pressure differential that pushes the ink through the open nozzles. Similarly, piezoelectric materials provide a pressure difference to eject ink, however they use piezoelectric crystals that change shape when a potential is applied. The resolution of inkjet printing is limited to the diameter of a stable ejected droplet (conventional material printers ~50 μm). The height of the printed materials can be adjusted by printing consecutive layers on top of one another, often times carbon-based materials require over 20 printed passes to be adequately conductive.

Inkjet printing require careful tailoring the surface tensions and viscosity of the ink to achieve stable droplets after ejection. Additionally, to achieve proper surface morphology, the wetting and drying properties of the ink must also be tuned to prevent unwanted coffee ring effects. Moreover, the size of the particles needs to be small and the solvents non-volatile to prevent clogging of the inkjet printhead, but the carbon material concentration should be high in order to form conductive patterns. Secor et. al. developed an ethyl cellulose graphene ink
(3.5 mg mL\(^{-1}\)) using cyclohexanone/terpineol.\(^{84}\) The fabricated ink was demonstrated to print line resolutions down to 50 µm, Figure 2.3c. We have demonstrated inkjet printing of a similar graphene ink to print disc electrodes used for hydrogen peroxide sensing and glucose sensing\(^{23}\) as well as ion selective potassium sensors.\(^{85}\) We have even demonstrated inkjet printing of interdigitated electrodes for electrical stimuli for differentiating stem cells.\(^{89}\)

**Inkjet Maskless Lithography**

The major limitation for inkjet printing is the narrow range of inkjet printable fluid properties (e.g. viscosity, density, surface tensions, particle size, etc…). Recently we have developed a new manufacturing technique called Inkjet Maskless Lithography (IML) which incorporates the versatility and rapid prototyping ability of inkjet printing but improves on its resolution.\(^{22},\,^{90}\) This template-free method is accomplished by a four-step printing process (Figure 2.3d). First, a polymer pattern which acts a sacrificial layer is inkjet printed in the negative pattern (designed in CAD software). Second, a highly concentrated solution-phase graphene ink (15 mg mL\(^{-1}\)) is spin coated over the substrate and polymer layer. Third, the graphene adhesion is increased through a post baking process in an oven or by heat gun. Finally, the sacrificial polymer layer is removed via solvent-based lift-off which removes the graphene not adhered directly to the substrate.

IML is able to print larger nanoparticles at higher concentrations with higher resolution and smaller feature size than conventional inkjet printing.\(^{90}\) We have demonstrated that IML is capable of patterning graphene printed lines with resolutions down to 20 µm while having very low resistance (~100 Ω sq\(^{-1}\)).\(^{22}\) As the pattern graphene is applied through spin coating, a more consistent thickness is able to be applied, instead of a dome-like deposition typically seen by inkjet printing.\(^{91}\) Moreover, IML printed graphene exhibits exceptional electroactivity and
high surface area making it an excellent transduction layer for electrochemical sensing. A pesticide biosensor using IML printed graphene and phosphotriesterase (PTE) demonstrated low detection limits (3 nM), high sensitivity (370 nA/µM) and had negligible interference from other pesticides.\textsuperscript{90}

**Aerosol-Jet Printing**

Similar to inkjet printing, aerosol-jet printing provides template-free versatility. This method atomizes droplets of inks and uses a gas stream to jet the ink at a substrate. Using a high frequency pressure wave (which are originated using an ultrasonic actuator), graphene ink is atomized into droplets. The ink mist is then carried by a gas to the deposition nozzles where it is focused by an addition inert gas. As much smaller ink droplets (~1-5 µm) are formed, higher resolution than inkjet printing can be obtained. Jabari et. al. formulated a concentrated graphene ink (60 mg/ml) with an ethyl cellulose binder in cyclohexanone and terpineol solvents.\textsuperscript{92} The research group was able to aerosol-jet print the graphene ink with widths down to 10 µm with sheet resistance as low as 1.64 kΩ sq\textsuperscript{-1}, Figure 2.3e.

**Laser Scribed**

Instead of directly patterning a solution-phase graphene ink, graphene can be pattern by reducing graphene oxide or carbonizing polymeric materials; this process is known as laser scribed graphene. Zhang et. al. developed this lithography-free technique to create graphene patterns by reducing graphene oxide films\textsuperscript{93} and later Lin et. al modified this method to carbonized polyimide films into graphene.\textsuperscript{94} Laser-scribed graphene presents an opportunity to print a new generation of disposable electrochemical sensors.\textsuperscript{95} McLamore’s group demonstrated that laser induced graphene can be fabricated into a flexible electrochemical sensor for sensing food spoilage (biogenic amines).\textsuperscript{96} Fenzl et. al. developed a highly sensitive
and reliable biosensor for blood serum analysis using laser-scribed graphene. They used 1-pyrenebutyric acid to immobilized an aptamer for selectively detecting thrombin (a coagulation factor). The sensor displayed low detection limits of 1 pM and was even demonstrated in complex bovine serum. Our group has similarly shown that laser-induced graphene is an excellent transduction material for flexible and disposable NH$_4^+$ and NO$_3^-$ sensor using ion-selective membranes. These sensors were tested in soil slurry and demonstrate accuracy within 5 percent of actual values.

Figure 2.3 Various graphene printing processes. a) Screen printing using a silicon stencil with line width down to 5 µm. Adapted with permission from (Hyun, et. al. Advanced materials 27(1)). Copyright (2014) Wiley Online Library. b) Gravure printing with line width down to 30 µm. Adapted with permission from (Secor, et. al. Advanced materials 26(26)). Copyright (2014) Wiley Online Library. c) Inkjet printing with line width down to 60 µm. Adapted with permission from (Qing, et. al. Applied materials and interfaces 9(17)). Copyright (2017) American Chemical Society and adapted with permission from (Secor, et. al. The journal of physical chemistry letters 4(8)). Copyright (2013) American Chemical Society. d) Inkjet maskless lithography line width down to 20 µm. Adapted with permission from (Hondred, et. al. ACS Nano 11(10)). Copyright (2017) American Chemical Society. e) Aerosol printing with line resolution down to 10 µm. Adapted with permission from (Jabari, et. al. Carbon 91). Copyright (2015) Elsevier.

Post Processing

As mentioned earlier, carbon inks typically include some sort of binder preventing unwanted particle aggregation, increase printability and improve morphology. Many of these
binders or surfactants (e.g. polymeric, epoxy, acrylic, cellulose) act as insulators which dramatically decreasing the conductivity of the printed carbon material. In order to increase the conductivity of the patterned graphene, post processing is often required.

**Thermal Annealing**

Thermal annealing is an effective method of increasing the conductivity of many carbon printed materials, Figure 2.4a. It effectively removes any remaining solvents, carbonizing surfactants (~300°C) and can “weld” individual flakes together (>500°C) which decreases electrical resistance. Thermally stable, flexible materials such as polyimide (T_g ~400°C) are able to increase to high temperature needed to carbonize surfactants, but most flexible substrate materials are not thermally stable (paper burns ~230°C, cotton degrades ~200°C, PET softens T_g ~ 70°C), so thermal annealing will destroy the substrate. Additionally, thermal annealing typically takes greater than an hour, adding manufacturing time to each device. To decrease the temperature necessary to anneal printed graphene, Secor et. al. developed a graphene ink using nitrocellulose (flash paper) which is largely removed at 200°C leading to electrical conductivity of ~10 000 S m$^{-1}$, Figure 2.4a. Upon increasing in temperature, surfactants continue to carbonize and graphene flake begin to “weld” together further increasing conductivity ~40 000 S m$^{-1}$, among the highest achieved for pristine graphene inks. The decrease in conductivity after 350 °C is due to the oxidation of the graphene surface as the high temperature thermal annealing was done in ambient air.

**Photonic Annealing**

Photonic annealing uses intense pulsed light (IPL) to rapidly heat the surface of the substrate. This process provides large-scale post processing of carbon inks by decomposing polymer ink stabilizers which increases the conductivity of the electrodes. Due to the large
disparity in optical absorbance between the substrate and carbon ink, rapid photothermal heating selectively occurs in the printed graphene without damaging the underlining substrate. Hersam’s group demonstrated that inkjet printed graphene can be rapidly post-processed using a pulsed xenon lamp on multiple different substrates: glass, PI, PEN and PET. The printed and annealed graphene exhibited excellent conductivity (~25 000 S m\(^{-1}\)) and less than 10% increase in resistance after 1000 bending cycles, Figure 2.4b.

**Laser Annealing**

Laser annealing provides a simple post processing step for annealing printed graphene by using a high-power laser (e.g. ND:YAG, CO\(_2\), Diode), illustrated in Figure 2.4b. Using high intensity lasers, irradiation energy quickly heats printed graphene (carbonizing surfactants, removing solvents, and “welds” graphene flakes together”) without damaging thermally sensitive and flexible substrates. Alternatively, laser annealing can also be used to directly reduce graphene oxides to pattern graphene. Contrary to thermal annealing or photonic annealing, laser annealing is not a whole substrate annealing process and can selectively anneal the printed graphene without damaging other printed components. For example, Del et. al demonstrated that drop casted graphene ink could be laser annealed to decrease its sheet resistance by 70%. We have shown that inkjet printed graphene can be annealed using a high-power third harmonic ND:Yag laser (15 ns pulse width) which dramatically increases the printed graphene conductivity without damaging the underlining flexible substrates (cellulose and polyimide). Using a laser power of 80 mJ cm\(^{-2}\) and 60 inkjet printed layers of graphene, the sheet resistance dropped to ~0.7 kΩ sq\(^{-1}\), Figure 2.4c. Similarly, we have shown that IML printed graphene on flexible PET can be laser annealed with a low-
cost (~$100) 1000 mW diode laser with similar results: a decrease in sheet resistance (roughly 3 orders of magnitude) and conductivity of ~26 000 S m$^{-1}$.

Figure 2.4 Printed graphene post-processing and annealing techniques. a) Thermal annealing. Adapted with permission from (Qing, et. al. Applied materials and interfaces 9(17)). Copyright (2017) American Chemical Society and adapted with permission from (Secor, et. al. Chemistry of Materials 29(5)). Copyright (2017) American Chemical Society. b) Photonic annealing, Adapted with permission from (Secor, et. al. Advanced materials 27(42)). Copyright (2015) American Chemical Society. c) Laser annealing. Adapted with permission from (Das, et. al. Nanoscale 8(35)). Copyright (2016) the Royal Society of Chemistry. d) Chemically reducing graphene oxide. Adapted with permission from (Mohan, et. al. Materials Science and Engineering 193). Copyright (2015) Elsevier.
**Chemical Reduction**

As graphene is more difficult to formulate into printed inks (aggregates, not easily dispersible in aqueous solutions), many researchers have instead formulated inks out of graphene oxide (heavily oxygenated graphene, bearing hydroxyl and epoxide functional groups on their basal plane). The presence of these functional groups makes graphene oxide highly hydrophilic and easily dispersible in aqueous solutions. After deposition, the insulating graphene oxide must be reduced into graphene to render the material electrically conductive. Stankovich et. al. first demonstrated that graphene oxide can be chemically reduced with organic isocyanates.\(^{100}\) Since then, researchers have reducing printed graphene for flexible electronics using a variety of chemicals, most commonly hydrazine vapor.\(^{101-102}\) For example, Mohan et. al. examined the chemical reduction of graphene oxide with three different hydrohalic acid reducing agents (hydroiodic acid, hydrazine hydrate, and hydrobromic acid).\(^{103}\) They found that hydroiodic reduction had the highest conductivity (~103 S cm\(^{-1}\)), while hydrazine and hydrobromic produced more defects which resulted in a lower efficiency in reduction, but potentially could be more electrocatalytic for electrochemical sensors.

**Surface Modification**

While electrochemical biosensors date back over fifty years (amperometric, enzymatic glucose sensor by Leland Clark in 1962)\(^{104}\) and have been commercially produced (glucose sensors, cholesterol, drug screening), their use has been limited due to low sensitivity and short shelf-life. With the recent advances in nanotechnology and the discover of new nanomaterials such as carbon nanotube, graphene, and metal nanoparticles, electrochemical biosensors have seen a dramatic increase in sensitivity and stability. Due to their large surface area to volume ratio, nanoparticles are able to dramatically increase the sensitivity and detection limits of
biosensors. Their nanometer features allow for enhanced substrate diffusion, increase working area for larger electrolyte interaction, and in many cases provide enhanced electroactivity. The surface of patterned solution-phase graphene can be modified to increase its electroactivity by: nanostructuring the surface to increase graphene’s edge planes and defect sites, electrodeposition metal nanoparticles for a higher surface/volume effect, doping the surface to add functional groups to enhance electroactivity or biorecognition binding, and increasing the porosity to increase graphene flake interaction with the solution for higher working area.

**Nano/Microstructuring**

While graphene theoretically has very high surface area (2630 m$^2$ g$^{-1}$), when patterned through typical solution-phase printing techniques the surface demonstrates low electrochemical surface area as the graphene compacts on the surface, exposing predominately their basal planes. In the context of electrochemistry, the basal plane of graphene exhibits much slower kinetics with four orders of magnitude lower specific capacitance and seven order lower electron transfer rates than the edge plane or defect sites in the graphene. Modifying the graphene surface by nano/microstructure the graphene flakes can improve the electroactivity of the graphene by revealing more edge planes and defect sites, as well as increase the electrochemical surface area.

While laser annealing is an effective tool for increasing the electrical conductivity of graphene, we have shown it can also significantly improves the electrochemical activity by converting 2D planar graphene into 3D graphene ‘petals’ with corrugated features. For example, using a Nd:YAG laser (15 ns pulse width) irradiation heating adds non-translational kinetic energy which creates lattice vibration. Tightly packed graphene flakes are able to
more rapidly dissipate the vibrations in the form of heat. Conversely, loose-packed flakes are able to vibrate at a higher rate and therefore do not dissipate as much heat, as seen in Figure 2.5a. The mismatch in vibration and thermal energy causes a lattice distortion changing 2D planar graphene to 3D petal-like graphene. Interestingly, we have seen a different effect using CO₂ laser (without pulsing) which does not tune the graphene flakes vertically, but rather etching through the basal planes of the graphene making nanosized (~100 nm) holes in the graphene lattice structure.¹⁰⁷

**Nanoparticle**

Electrodepositing nanoparticles, especially metal nanoparticles on carbon nanoparticle electrodes have shown enhanced electrocatalytic behaviors by increase current densities, faster mass transport due to convergent diffusion, more favorable faradaic-to-capacitive current ratios, and increase electrochemical surface area.¹⁰⁸ Hybridizing of nanoscale metals and with nano-carbon allotropes have shown to dramatically improve the electroactivity and are among the best-performing sensors to date.¹⁰⁹-¹¹² Furthermore, immobilizing biological agents onto the surface of nanoparticles can improve their activity and stability.¹¹³-¹¹⁴ We have previously developed a scalable nanostructured biosensor based on multilayered graphene petal nanosheets and electrodeposited platinum.¹⁰⁸ Combining zero-dimensional nanoparticles with a two-dimensional carbon transduction layer created an exceptional electrochemical biosensor for glucose monitoring. By varying the cathodic current for electrodeposition, the nanoparticle density, morphology, and size can be tailored, Figure 2.5b.

**Doping**

Carbon-based materials, graphene in particular, can be chemically, physically, and electroactively altered by doping. Chemical doping is the act of modifying the crystal structure
by including foreign atoms which have shown to enrich free charge-carrier density and enhance the electrical and thermal conductivity. Doping with nitrogen has shown to increase the biocompatibility and sensitivity of CNTs in biosensing applications. Researchers have wet-chemically p-doped graphene with nitric acid which decreased the sheet resistance of CVD grown graphene to 30 \( \Omega \text{ sq}^{-1} \). Wang et. al. successfully N-doped solution-phase patterned graphene with nitrogen plasma treating and increased its hydrogen peroxide sensitivity by ~20 times and developed a low detection (0.01 mM) glucose sensor. We have shown a similar technique of N-doping graphene by simply annealing inkjet printed graphene in a nitrogen environment and developing a potassium ion selective electrode with a limit of detection of \( \text{Log } K^+ = 10^{-5.2} \) with a linear sensing range of (0.01-10 mM).

**Porosity**

Increasing the porosity of the pattern graphene can also significantly improve the electroactivity by providing addition oxidation/reduction sites which increases the electrode working area. Moreover, having a dense porous structure creates a large scaffold area for immobilizing biological agents allowing the substrate to diffuse into the electrode and react with the biological species. One simple process to microstructure and tailor the porosity of graphene is polymer-phase inversion. Choi et. al. demonstrated the use of Nafion into the graphene solution which increased the hydrophobicity to over 160° due to the petal-like structure with hierarchical roughness. Secor et. al tailored the graphene porosity using glycerol which led to phase-separation and upon drying pore-formation. The porosity of the printed graphene can be varied by the amount of glycerol used, Figure 2.5c: left. Unfortunately, increasing the porosity of solution-phase graphene using phase-inversion typically decreasing the conductivity of the graphene due to high flake-to-flake resistance.
Similarly, we have enhanced the electroactive surface of IML graphene by increasing the porosity and edge plane defects sites through construction of a multidimensional architecture via salt impregnated inkjet maskless lithography (SIIML) and CO$_2$ laser annealing. This method uses discretely sized salt crystals that act as hard templates for pore formation and which are later dissolve in water making macroscale pores, Figure 2.5c: right. Subsequently a CO$_2$ laser etches the surface making microsized pores (~100 nm) and defects in the lattice structure. Unlike polymer-phase inversion, SIIML does not increase resistivity, but actually increases the conductivity nearly three orders of magnitude (~10 000 Ω sq$^{-1}$ to ~50 Ω sq$^{-1}$), due to the simultaneous laser annealing. Using SIIML a sensitive pesticide biosensor using ACHE was developed which was over two times as sensitive to thiocholine than patterned graphene without macropores.

![Figure 2.5 Graphene surface modification.](image_url)

Figure 2.5 Graphene surface modification. a) Molecular dynamic simulation of laser annealed graphene showing nano/microstructuring mechanism. Adapted with permission from (Das, et. al. *Nanoscale* 8(35)). Copyright (2016) the Royal Society of Chemistry. SEM showing graphene surface before and after laser annealing. Adapted with permission from (Hondred, et. al. *ACS Nano* 11(10)). Copyright (2017) American Chemistry Society. b) Electrodeposition
of nanoparticles. Adapted with permission from (Claussen, et. al. *Advance Functional Materials* 22(16)). Copyright (2012) Wiley Online Library. c) Increasing the graphene porosity through polymer phase inversion. Adapted with permission from (Secor, et. al. *The Journal of Physical Chemistry C* 122(25)). Copyright (2018) Wiley Online Library. Increasing porosity through SIIML. Adapted with permission from (Hondred, et. al. *Nanoscale Horizons*). Copyright (2019) the Royal Society of Chemistry.

**Immobilization/Functionalization**

While there are a variety of different immobilization strategies able to functionalize biological recognition agents for electrochemical biosensors, a couple of strategies work efficiently well for carbon materials. The most basic strategy is physical adsorption which uses physical interactions generated between the carbon transduction material and the biological protein.\(^{121}\) The binding is relatively weak and often not very effective. Another immobilization strategy is the use of a matrix to bind the biological material to the surface such as through a nafion polymer matrix or glutaraldehyde, Figure 2.6a.\(^90\) Binding through crosslinking or a matrix, while simple and effective, often blocks the surface of the electrode or binding pockets of the biological agent. Covalent bonding, especially though EDC/NHS (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide) chemistry is one the most popular method of functionalization to carbon materials due to the many carboxyl groups typically found on the surface of many carbon electrodes, Figure 2.6b.\(^{120}\) EDC/NHS covalently bonds a biological species to the surface with a “zero-length” crosslinking agent which couples a carboxyl to a primary amine.\(^{122}\) Ionic interactions can be accomplished through the use of nanoparticles or metal ion amino acids on the surface of the carbon transduction layer, Figure 2.6c.\(^{105}\) For example, Stanciu developed an electrochemical pesticide sensor by functionalizing acetylcholinesterase to graphene oxide using histidine’s metal affinity towards nickel.\(^{123}\) Finally, biological molecules can be functionalized through weak van der Waals and electrostatic forces using π-π interaction (e.g. pyrene) and CH–π interactions (non-aromatic
molecules, e.g. pentane). These non-covalent attachment mechanisms do not require graphene functional groups or even alter the graphene lattice structure, making them an attractive option for CVD graphene transistor biosensors. However, the non-covalent immobilizations provide weak interactions which often provide less stability.

Figure 2.6 Common graphene functionalization schemes: a) crosslinking via glutaraldehyde. Adapted with permission from (Hondred, et. al. ACS applied materials and interfaces 10(13)). Copyright (2018) American Chemical Society. b) ionic binding by histidine’s metal affinity interaction. Adapted with permission from (Hondred, et. al. Analyst 142(17)). Copyright (2017) the Royal Society of Chemistry. c) Covalent binding through EDC/NHS. Adapted with permission from (Hondred, et. al. Nanoscale Horizons). Copyright (2019) the Royal Society of Chemistry.
Applications

Wearable Physiological Monitoring

One of the most common applications for flexible electrochemical biosensors is to be integrated into a device and/or worn on the body, referred to as wearables. Flexible electrochemical biosensors are especially important for wearable electronics as they can provide real-time feedback information regarding the wearer’s health, performance, vitals, or environment information to the user and have been employed for a variety of different fields including healthcare, entertainment and security. They can be directly integrated onto or into clothes, adhered to the skin as a temporary tattoo or worn as a garment. Unlike standard wearable electronics which monitor physical activity or vital statues (e.g. temperature, motion, ECG, blood pressure), biosensors can continuously monitor the chemical and biochemical vitals though bodily fluids. Sweat, saliva and tears provide a plethora of information for health monitoring and diagnostics such as physiological metabolites (glucose, lactate, cortisol, adrenaline), tissue therapeutics for wound repair, and electrolyte balance. Additionally, wearables can monitor environmental conditions such as environmental toxicity due to heavy metals, pesticides, or chemical warfare weapons. Carbon-based electronics provide a flexible conductive material and high electrochemical surface area needed for wearable biosensors. Additionally, it is an attractive material for wearables as it typically has little irritation to skin (although reference material such as Ag/AgCl has shown minor dermal irritation).
Textile

Continuous monitoring wearable biosensors, which seamlessly integrate into a person’s daily routine, are becoming a major area of study. They can provide remote monitoring (for patient telecommunication), monitor environmental and vital signs of military personnel, and can provide real-time physical conditions for athletes. Textile wearable sensors provide the attractive advantage of integrating into a person’s clothes. Guinovart et. al demonstrated a simple approach to designing and fabricating a sensitive electrochemical biosensor using CNT modified cotton yarn using an ion-selective membrane for sensing pH, K\(^+\) and NH\(_4^+\), Figure 2.7a. A cotton yarn was dipped in a CNT ink multiple times and rinsed with DI water to remove surfactants and coated with an ionophore. The final CNT yarn had a resistance of approximately 500 Ω cm\(^{-1}\). The designed electrochemical biosensor displayed analytical performance similar to lab-made electrodes, limit of detections ~10 µM for K\(^+\) and 1 µM for NH\(_4^+\). This wearable sensor was fabricated into a band-aid device, but as the sensor is a cotton fiber, it could potentially be fabricated directly into smart clothing. Wang’s group reported the first textile-based electrochemical biosensor which was integrated directly into clothing, Figure 2.7b. An amperometric screen-printed carbon electrode was incorporated into brief underwear (cotton substrate) as it provides direct, tight, and intimate contact with the epidermis. Even under repeated folding (180°) and stretching stresses that would arise from normal wear of clothing, the textile screen-printed carbon electrode did not crack and show repeatable electrochemical responses.

Tattoo

While textile-based biosensors provide some physical contact with the skin, they have limited bodily uses as many areas of the body do not have tight contact with clothes. Flexible
polymer-based electrochemical patches, such as tattoo films, can be applied to any bare skin area of the body. As tattoo based electrochemical biosensors are in direct contact with the skin, they require unique mechanical and physical requirements in order to match the epidermis complex bending and stretching stress. The first example of a tattoo-based electroanalytical sensor (potentially biosensor) was developed by Windmiller et. al, Figure 2.7c. The temporary transfer tattoo was fabricated by screen printing a carbon/carbon fiber ink on tattoo polymer substrate. The carbon fiber has multiple purposes in the ink such as reinforcing the printed transduction layer for increase mechanical resilience, decreases electrical resistance by acting as a conductive backbone, and improving the electrochemical activity for improved sensitivity. The tattoo electrochemical sensor demonstrated excellence mechanical resilience to extreme deformation and show consistent uric acid sensing even after multiple washing cycles. Similarly, Wang’s group designed the first real-time wireless lactate sensor using a printed flexible electrochemical biosensor, Figure 2.7d. Lactate is an important biomarker in tissue oxygenation and therefore can be used for physical performance metrics for sports, military, and health care applications. The researchers immobilized Lactate oxidase on a screen-printed carbon fiber electrode on a temporary tattoo polymer film. Upon exercise the tattoo biosensor recorded dramatic increase in amperometric current on the functionalized electrode but the control electrode (no enzyme) remained relatively stable, demonstrated its sensitivity to lactate.

**Non-Clothes Accessories**

Wearables accessories that are not textile based provide routes to monitoring different bodily fluids other than just sweat. They do not need to be integrated into other clothes or textiles as they are fabricated as their own device. Wang’s group developed a wearable salivary
metabolite biosensor using printed technology on a mouthguard, Figure 2.7e. 132 The biosensor was constructed by screen printing prussian-blue carbon paste on flexible PET substrate and entrapping lactate oxidase by electrodepositing poly-orthophenylenediamine (PPD). The flexible biosensor was then adhered to the mouthguard with double sided adhesive tape. The biosensor had a limit of detection ~50 µM and a sensitivity of 0.5 µA mM\(^{-1}\). The fabricated mouthguard biosensor was able to detect lactate in saliva within 10% error. The group later expanded upon this design by integrating a potentiostat, microcontroller, and a Bluetooth low energy transceiver, which allows for real-time wireless transmission of the sensed information to a smartphone or laptop.133

Smart watches, wrist bands, or exercise arm straps are a common wearable accessory that many people often wear in everyday settings. Incorporating electrochemical biosensor into these devices provide valuable information especially towards health or exercise monitoring. The structural device also provides useful space for incorporating electronics (circuitry, Bluetooth, potentiostat, etc…) that most wearables do not have. Diamond’s group developed a biosensor sweat watch (“Sweatch”) that integrates all components into a small compact wearable Velcro arm band, Figure 2.7f.134 The sweatch incorporated a screen printed carbon electrode on flexible PET substrate and was modified with solid-state ion selective ionophore. An absorbent material was used to collect the sweat and wick it to the functionalized electrodes where the ionophore biosensor was able to selectively detect sodium concentrations. The biosensor had a linear sensing range from \(10^{-4}\) to \(10^{-1}\) M and was able to detect sodium concentration (~44 mM) during exercise which was consistent with other reported exercise sweat concentrations.
Figure 2.7 Carbon-based wearable electrochemical biosensors. a) Carbon nanotube fiber textile Band-Aid biosensor. Adapted with permission from (Guinovart, et. al. Analyst 138(18)). Copyright (2013) American Chemical Society. b) Screen printed carbon paste electrode underwear biosensor. Adapted with permission from (Yang, et. al. Analyst 135(6)). Copyright (2010) American Chemical Society. c) First tattoo electrochemical sensor using a carbon nanofiber ink. Adapted with permission from (Windmiller, et. al. Chemical Communications 10(13)). Copyright (2012) Royal Society of Chemistry. d) Screen printed carbon ink tattoo biosensor for lactate monitoring. Adapted with permission from (Wenzhao, et. al. Analytical Chemistry 85(14)). Copyright (2013) American Chemical Society. e) Screen printed carbon mouthguard biosensor for metabolic sensing. Adapted with permission from (Kim, et. al. Analyst 139(7)). Copyright (2014) American Chemical Society. f) Screen printed carbon arm band biosensor for salt detection in sweat. Adapted with permission from (Glennon, et. al. Electroanalysis 28(6)). Copyright (2016) Wiley Online Library.
Origami

Flexible substrates such as paper provide an attractive low-cost method for fabricating inexpensive, biodegradable, and foldable biosensor. Using origami (process of folding paper into shapes or figures), electrochemical paper-based biosensor can be folded into hierarchical structure with multiple layers out of one single continuous substrate. These multiple layers can be used for multiple different purposes, but the main one is for use with microfluidic flow cells. Additionally, the multilayered structure can be used to separate different enzymes, substrates and samples, and when folded combined all components together for sensing. Liu et. al. developed an origami paper biosensor with screen printed carbon and wax printed channels that operates with a digital multimeter. The biosensor utilizes an aptamer for adenosine detection (a biological cofactor found in kidney function) with a detection limit of 11.8 µM and a sensitivity of 0.48 µA µM$^{-1}$. Additionally, they used the microfluidic flow cell as a fluorescent adenosine aptamer sensor which showed a greater fluorescence when adenosine was present, Figure 2.8a. Similarly, Yu’s group developed a multilayered origami biosensor with screen printed carbon electrodes (Figure 2.8b) and modified with graphene and gold nanoparticles. The fabricated biosensor showed effective sensing for ssDNA with a limit of detection of $2 \times 10^{-16}$ mM. The sensor also performed well in human serum and can be easily applied for point-of-care testing to detect target DNA in complex medium. Wang et. al use origami to design a “pop-up” electrochemical paper-based biosensor that detects beta-hydroxybutyrate, a biomarker for diabetic ketoacidosis, using a commercial glucometer, Figure 2.8c. The 3D structure which can be folded and unfolded makes it possible to change the fluidic path, electrical contact paths, and to control timing events. The origami biosensor was constructed on cellulose paper and stencil-printed graphite ink electrodes. As the sensor can be folded, fluidic paths can be manually timed, and an enzyme/substrate incubation step
could be included. The concentration of enzyme (3-hydroxybutyrate dehydrogenase ~$6/U) was able to be reduced due to the added incubation step which dramatically decreased the cost per device.

**Wireless Biosensors (RFID)**

While biosensors can be designed and printed into small electrodes on a variety of different substrates for flexible and wearable devices, they usually need to be directly connected to a potentiostat with conductive wires to power and monitor electrical responses. The need for wires can significantly impede the usefulness of electrochemical sensors, especially wearable biosensors. One solution to this problem is to use radio frequency identification (RFID) or near field communication devices. RFID biosensors use an inductance coil and an impedance biosensor capacitor. An antenna sends a radio frequency and the inductance coil of the RFID biosensor sends a resonating frequency back. When the bioreceptor binds with the target analyte, the capacitor changes impedance which in turn changes the resonating frequency of the RFID biosensor. Tanguy et. al. demonstrated the use of a custom designed 13.56 MHz RFID biosensor for selectively detecting small amounts of putrescine (common biogenic amine produced during food spoilage), Figure 2.8d.

They used a passive RFID tag modified with a carbon paste working electrode composed of MWCNTS and immobilized maleic anhydride in a polymeric matrix. The RFID biosensor was able to selective detect putrescine down to 250 mM. The biosensor performance was increased 2x by functionalizing the sensor using a polymer binder and operated over 40 percent faster when the working electrode was modified with MWCNTs over just carbon paste. RFID biosensors can also be fabricated for physiological monitoring and worn on the body; for example, McAlpine’s group developed a passive, wireless graphene RFID biosensor. Graphene was
first printed on water-soluble silk substrate and then transferred onto a interdigitated electrode connected to an inductance RFID coil. The electrochemical biosensor device can then be attached to a tissue or even tooth enamel, Figure 2.8e. The resulting biosensor is capable of extremely low detection limits, even down to a single bacterium while wirelessly transmitting the information. Kim et. al design a smart contact lens for wirelessly monitoring glucose and intraocular pressure (risk factors associated with diabetes and glaucoma), Figure 2.8f.\textsuperscript{143} The biosensor and pressure sensor was fabricated into field effect transistors (FET) biosensors using a graphene-silver nanowire hybrid structure that was stretchable and optically transparent. Glucose oxidase was then immobilized using a pyrene linker to the graphene on the FET. This FET was connected to an inductance coil for RFID for wirelessly monitoring. The RFID biosensor was able to measure glucose in tear fluid with a resonating frequency shift of 4.1 GHz. Furthermore, the contact biosensor was tested on a rabbit eye. The rabbit was fed, and the contact RFID biosensor was able to monitor an increasing in glucose and no signs of abnormal behavior while the rabbit was wearing the lens.

While there has been significant interest in RFID biosensor for wireless and passive monitoring, the inductance coil has predomently been made of patterned or printed metals as very low resistance ($<5 \ \Omega \ \text{sq}^{-1}$) is necessary to establish a strong resonating frequency. Improving the conductivity of printed graphene could have dramatic effects on lowering the cost of printed RFID biosensors and provide a biofriendly/biodegradable device. Leng et. al. demonstrated that such printed graphene antennas can be fabricated, Figure 2.8g.\textsuperscript{144} Their group used a graphene nanoflake conductive ink that was screen printed on cellulose paper substrate and used compression rolling to increase the conductivity of the printed graphene. The graphene film was compressed from $\sim30 \ \mu\text{m}$ to $6 \ \mu\text{m}$ and the sheet resistance droped for
While the RFID was not constructed into a biosensor, it provided the bandwidth, gain, and radiation required for mid- and short-range RFID and sensing applications using printed graphene ink on flexible and low-cost paper-substrate.

**Disposable Biosensors**

Flexible carbon-based electrochemical biosensors provide economical test strips which dramatically lower the cost per device making the price point feasible for a single use disposable biosensor (e.g. most glucose biosensor test strips). Manufacturing biosensors using carbon-based materials such as graphene, versatile printing techniques, and flexible substrates allows for mass production roll-to-roll printing process. The biofriendly carbon material not only provides a conductive transduction layer for electronic circuitry but provides a biocompatible surface for biological functionalization that is electrochemically active. Su et al demonstrated a dopamine biosensor by chemically reducing graphene oxide film by inkjet printing ascorbic acid onto a graphene oxide film with a PET flexible substrate. Using this “inkjet reduction” technique an interdigitated electrode was developed, Figure 2.8h. The graphene IDE dopamine sensor displayed great flexibility, low-cost, and high sensitivity (limit of detection of 1 µM). We have shown that laser induced graphene can be fabricated into nitrogen soil sensors. This method provides very low cost and flexible sensors as solution-phase graphene is not required, Figure 2.8i. We have also shown that large patterns can be printed using IML to fabricate sensitive pesticide or chemical warfare disposable biosensors on PET using Organophosphate Hydrolase or Acetylcholinesterase. The pesticide biosensor has very low detection limits (0.6 nm) with high sensitivity (12.4 nA nM⁻¹) to paraoxon, a model organophosphate pesticide, Figure 2.8j.
Figure 2.8 More applications of flexible carbon-based electrochemical biosensors. a) Origami microfluidic fluorescent and electrochemical biosensor. Adapted with permission from (Liu, et. al. Angewandte chemie international edition 51(28)). Copyright (2012) Wiley Online Library. b) Foldable point of care DNA biosensor. Adapted with permission from (Lu, et. al. Electrochimica acta 80). Copyright (2012) Elsevier. c) “Pop-up” biosensor. Adapted with permission from (Wang, et. al. Analytical chemistry 88(12)). Copyright (2016) American
Printed all-carbon biosensor also provides a disposable test strip that will not pollute the environment with heavy metal toxicity. Additionally, some biosensors have even been fabricated on biodegradable substrates that break down over time into environmentally acceptable constituents such as carbon dioxide and water. For example, we have demonstrated that inkjet printed graphene on nanocellulose and functionalized with glucose oxidase can be used a low-cost biodegradable test strip for glucose sensing, Figure 2.8k.Using the versatility provided by inkjet printing, intricate patterns that can improve the diffusion rates of substrate to the electrode surface enhancing signal/noise ratios was also shown. Wu et. al. used disposable cellulose substrate (filter paper) and screen-printed carbon ink to design a flow-cell electrochemical multiplex biosensor, Figure 2.8l. By including graphene on the surface of the working electrodes, the efficiency of the immunodevice was increased by enhancing the electron transfer. The biosensor was able to detect multiple different cancer biomarkers (Human AFP, CEA, CA125, and CA153) with a detection limit below a pg mL$^{-1}$. 
Challenges and Future Work

Flexible nano-carbon allotrope printed electrochemical biosensors are still at their infancy, but they have experienced tremendous growth over the last couple of years due to groundbreaking research and discoveries in material science, manufacturing/printing technology, biochemistry, and innovative applications of nano-carbon allotropes. The discovery of new carbon-based materials such as CNTs and graphene has ushered the way for flexible printed electronics that possess high surface area, biocompatibility and enhanced electroactivity. Development of new printing methods, post processing methods and surface enhancement have improved the resolution, conductivity and sensitivity of electrochemical biosensors. However, there are still many challenges to overcome before printed carbon-based electrochemical biosensor are commercially produced and widely accepted.

First, the majority of graphene biosensor research (even many of the applications described in this manuscript) has been limited to modifying the working electrode for enhanced electrochemical activity, few researchers have explored the use of graphene for full electrode manufacturing without the use of a metallic printed conducting material. Developing fully graphene electronics that are printed could reduce manufacturing costs, increase biodegradability, reduce environmental toxicity when disposed, and improve sensor performance. Graphene could potentially outperform metal electronics as they are 2D materials that can bend and stretch at a higher degree.

Second, while material printing processes and carbon ink formulations have dramatically improved over the last few years, further research to increase the conductivity, printability and electrode mechanical stability must be improved. Developing new ink binders that decrease the printed graphene flake-to-flake resistance and improve the conductivity are
one the major hurtles preventing carbon-based inks from being industrially used. Many applications require extremely low sheet resistance (such as RFID inductance coils). Additionally, research into more flexible and even stretchable printed carbon materials and fillers would provide more mechanical applications making them a more attractive choice than metal electrodes. There should be further research into using conductive polymer/elastomeric binders which provide the printed carbon material enhanced stretchability without decreasing conductivity.

Third, current printing methods are inadequate for depositing carbon materials with high resolution and small feature size. We have discussed many printing techniques which have deposited graphene with high resolution and feature sizes down to 10s of microns. However, these printing methods are dependent on the physical properties (porosity, surface tensions) of material they are patterning onto. Additionally, graphene and CNT ink have the unfortunate effects of clogging nozzles, grooves, or wells which provide inconsistencies. Many biosensors such as interdigitated electrodes require very high resolution with small feature size (sub-micron) to boost their performance which current printing technology is unable to obtain.

Fourth, while electrochemical biosensors have been around for years, there has been little adoption into our present society (apart from glucose sensors). There are multiple different reasons for this sluggish acceptance such as stability, reproducibility, low sensitivity, and longevity issues with biosensors. Due to the unique properties of graphene that we have previously discussed, graphene biosensors have shown increase sensitivity and stability, but further research into improving immobilization strategies and biological recognition efficiency is needed.
Finally, providing power and connecting biosensors to potentiostats have been a critical challenge for flexible and especially wearable biosensors. Significant research has been conducted in recent years to decrease the size of potentiostats and providing wireless transmission (e.g. Bluetooth), but much more research into integrating these electronics into conform fitting or integrated wearable packaging is needed. The incorporating of RFID have had a significant impact as the biosensor do not need to be directly powered and can wireless transmit data through resonating radio waves, but these sensors are limited to impedance changes. As RFID biosensor do not apply power, a potential for oxidation/reduction mechanism cannot be employed which removes most enzymatic biosensors. Developing new, compact, and integrated ways of transmitting data wirelessly could significantly improve societies acceptance of wearable and flexible electrochemical biosensors.

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CHAPTER 3. ENHANCED ENZYMATIC ACTIVITY FROM PHOSPHOTRIESTERASE TRIMER GOLD NANOPARTICLE BIOCONJUGATES FOR PESTICIDE DETECTION

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Abstract

The rapid detection of organophosphates (OPs), a class of strong neurotoxins, is critically important for monitoring acute insecticide exposure and potential chemical warfare agent use. Herein, we improve the enzymatic activity of a phosphotriesterase trimer (PTE₃), an enzyme that selectively recognizes OPs directly, by conjugation with distinctly sized (i.e., 5, 10, and 20 nm diameter) gold nanoparticles (AuNPs). The number of enzymes immobilized on the AuNP was controlled by conjugating increasing molar ratios of PTE₃ onto the AuNP surface via metal affinity coordination. This occurs between the PTE₃-His₆ termini and the AuNP-displayed Ni²⁺-nitrilotriacetic acid end groups and was confirmed with gel electrophoresis. The enzymatic efficiency of the resultant PTE₃-AuNP bioconjugates was analyzed via full enzyme progress curves acquired from two distinct assay formats that compared free unbound PTE₃ with the following PTE₃-AuNP bioconjugates: 1) fixed concentration of AuNPs while increasing the bioconjugate molar ratio of PTE₃ displayed around the AuNP and 2) fixed concentration of PTE₃ while increasing the bioconjugate molar ratio of PTE₃-AuNP by decreasing the AuNP concentration. Both assay formats monitored the absorbance of p-nitrophenol that was produced as PTE₃ hydrolyzed the substrate paraoxon, a commercial insecticide and OP nerve agent simulant. Results demonstrate a general equivalent trend between the two formats. For all experiments, a maximum enzymatic velocity (V_max)
increased by 17-fold over free enzyme for the lowest PTE3-AuNP ratio and the largest AuNP (i.e., ratio of 1:1, 20 nm dia. AuNP). This work provides a route to improve enzymatic OP detection strategies with enzyme-nanoparticle bioconjugates.

**Introduction**

Organophosphates (OPs) are widely used as neuroinhibitory pesticides to prevent crop loss. However, OPs pose a significant risk to the environment through water run-off and an even greater risk to humans in the form of nerve agents that are used in chemical weapons (e.g., Soman and Sarin). OPs released into the environment degrade with time, but such degradation has not prevented trace levels appearing in drinking water supplies. Therefore, there is a strong need to produce sensors that can rapidly assess and continuously monitor exposure/contamination from OP-based pesticides and chemical warfare/terror agents before such chemicals have the opportunity to leech deep into the soil. Enzyme-based biosensors display tremendous promise for rapid, continuous, and low-cost biosensing needed for in-field OP sensing.

Several enzymes have been explored for enzymatic OP sensing including organophosphate hydrolase (OPH). OPH is a lipoprotein that hydrolyzes the triester bond of OP pesticides such as paraoxon, methyl-parathion and diazinon and has been shown to hydrolyze paraoxon rapidly - paraoxon breakdown by OPH appears to approach the diffusion limit of the substrate ($V/K_M \approx 10^8-10^9 \text{ M}^{-1}\text{s}^{-1}$). Phosphotriesterase from *Brevundimonas diminuta* is an OPH variant of specific interest as it is able to selectively target triple O-linked phosphonate centers such as those found in Sarin and Tabun. Multiple derivative OPH structures, including a de novo chimeric collagen-PTE trimer (PTE3), have been engineered to increase the enzyme’s activity within very specific and targeted applications. However, the reaction kinetics and stability of
OPH and some of its derivatives are currently not considered sufficiently robust nor sufficiently sensitive for use in portable, field-deployable OP biosensing devices.

For decades, researchers have been exploring possible solutions for increasing the stability and activity of OPH with diverse immobilization techniques. For example, protein fusion, which anchors OPH onto the surface of *Escherichia coli* using an Lpp-OmpA fusion protein, demonstrated a seven-fold increase in parathion degradation.\(^\text{15}\) OPH entrapment within a polyurethane foam matrix provided increased enzymatic life (1.8 days soluble and 278 days immobilized when stored at 25°C) and thermal stability (1.5 hours soluble and 158.4 hours immobilized at 50°C).\(^\text{16}\) Researchers have also demonstrated that OPH retained enhanced enzymatic stability when attached to nanomaterials such as silica matrices (no significant loss after 12 months),\(^\text{17}\) amyloid fibrils (300% increase in relative temperature stability at 40, 45, and 50°C),\(^\text{18}\) carbon nanotubes (only 25% signal loss after 7 months),\(^\text{19}\) and nanoparticles (NPs) such as nanocrystalline semiconductor quantum dots (QDs).\(^\text{20}\) The latter provide biocompatible surfaces, potential for enhanced conductivity through direct enzyme electrical ‘wiring’,\(^\text{21}\) and high surface area for immobilization of enzymes. In this vein, we recently demonstrated that PTE\(_3\) has enhanced activity when displayed on CdSe/ZnS core/shell QDs.\(^\text{20}\) Indeed, recent research has shown that enzyme immobilization on NPs in general can increase both the sensitivity and stability of enzymes for a variety of applications.\(^\text{22-24}\)

While the exact interaction and catalytic nature between NPs and enzymes are complex and much is still unknown, the specific characteristics of enzyme-NP conjugates such as mass transport, enzyme orientation, surface morphology, and enzyme density have been shown in many cases to enhance enzymatic performance.\(^\text{24-26}\) NPs have unique properties of high surface-to-volume ratios as well as high radii of curvature which can potentially allow enzymes
to be positioned with increased distance between adjacent immobilized enzymes; this may limit unfavorable protein to protein interaction(s) on the NP surface. Enzyme-NP bioconjugates can be further manipulated by changing the attachment chemistry of the enzyme in order to situate the enzyme’s binding pocket away from the NP while optimizing the position for substrate to diffuse to and from the enzyme. Enzymes that have been immobilized onto the surface of NPs display improvements in activity along with a wider performance window across a range of pH and temperature changes. These advantages have not only provided enhanced enzyme activity, stability, and specificity, but have also improved the ability of enzymes to be used in sensors for a variety of conditions such as for the highly sensitive detection of glutamate, glucose, lactate in simulated or actual biological solutions/conditions. Moreover, we have shown that immobilizing the enzyme alkaline phosphatase onto QDs can improve the enzymatic efficiency ($k_{cat}/K_M$) up to 40% versus free enzyme. We have also shown that immobilizing trypsin substrate onto NPs can enhance enzymatic activity in Förster resonance energy transfer (FRET) assays where the rate of trypsin-catalyzed proteolysis of QD-displayed peptide increased to five times that of free enzyme. AuNPs have shown enhanced effects on a variety of small molecules such as improved affinity for Alzheimer’s peptide Aβ (a factor of 7 higher for NP-immobilized D3 than for the free ligands) and anti-viral lectin Cyanovirin-N (several orders of magnitude higher than isolated monomeric sugars interacting with the lectin), increased enzyme selectivity of α-chymotrypsin (~3-fold improvement for cationic substrates), and lowered $K_m$ values of glucose oxidase (1.56-fold lower in magnitude than free glucose oxidase); however, the enhanced enzymatic performance of OPH using AuNP has not been researched. Here, we improve the performance of PTE$_3$ via immobilization onto gold nanoparticles
(AuNPs). PTE$_3$ is attached to the AuNP via metal-affinity interactions allowing for ratiometric and orientation control on the AuNP.$^{39}$ This method orients the catalytic site on the outside of the bioconjugate increasing the likelihood of enzyme/substrate binding to occur.$^{40}$ PTE$_3$ was immobilized onto 5, 10, and 20 nm diameter AuNPs in an effort to analyze trends between enzyme activity and NP carrier size/curvature along with immobilized enzyme density, see Figure 3.1. In this work, the organophosphate paraoxon was used as substrate since its hydrolysis produces p-nitrophenol which has a distinct, measurable absorbance (405 nm)$^{14}$ PTE$_3$-AuNP performance was monitored in two distinct formats: fixed amounts of AuNPs where the molar ratio of PTE$_3$ per AuNP was systematically increased and fixed concentrations of PTE$_3$ where the amount of AuNPs were systematically decreased to increase the ratio of PTE$_3$/AuNP. The data again confirms enzymatic enhancement of PTE$_3$ when displayed on NP display and that this can be extended to other types of NP materials with different sizes utilizing different enzyme attachment chemistries.

**Methods and Materials**

**Chemicals**

Paraaxon was purchased from Chem Services (USA). All other chemicals including solvents were purchased from Sigma-Aldrich (USA) or Acros Organics (USA), unless indicated otherwise, and used as received without any other further purification unless stated. The use of these chemicals are outlined in the following sections of this Experimental Methods section.

**PTE$_3$ Expression**

Briefly, the gene cassette encoding the phosphotriesterase gene (PTE, EC 3.1.8.1) from *Brevundimonas diminuta* and the collagen-like protein from *Streptococcus pyogenes* was
synthesized by Genescript. The multimerization domain consisted of the V-domain which facilitates assembly of the structure and a collagen helix comprised of 78 repeats of the glycine trimer (Gly-Xaa-Yaa). The cassette was flanked by unique, terminal restriction enzyme cleavage sites which facilitated cloning into either the cytoplasmic or periplasmic pET bacterial expression plasmids (Takara Bio, USA). Integration into either expression construct allowed for the addition of a C-terminal hexahistidine tag (His$_6$) that is used for purification of the protein with immobilized metal affinity chromatography (IMAC). Additional details on vector construction can be found in our previous work.$^{14}$

Expression of the PTE$_3$ was performed in *E. coli* strain BL21(DE3). Briefly, 500 mL of Terrific Broth containing the appropriate antibiotic was inoculated with 5 mL of an overnight culture. The culture was maintained at 37°C for 3 hours or until mid-log phase was reached. Expression of the recombinant protein was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.25 mM. The culture was maintained at 30°C for 15-24 hours then centrifuged at 4000 x g to pellet the cells. The resultant cell pellet was frozen at -80°C for a minimum of 3 hours to aid in cell lysis. Following the incubation at -80°C, cells were resuspended in 30-50 mL of lysis buffer (0.5X phosphate buffered saline – PBS, 69 mM NaCl; 1.4 mM KCl, pH 7.4), 1 mM ethylenediamine tetraacetic acid (EDTA), 0.1% Triton X-100, 2 mg/mL lysozyme) and incubated on ice for 30 minutes with constant, slow agitation. The cell slurry was then sonicated 6 times at 30 second intervals using a Branson Sonifier (constant output, duty cycle 5). Soluble and insoluble material was separated via centrifugation at 15000 x g for 30 minutes. Soluble material was decanted to a fresh conical tube and combined with Ni$^{2+}$-nitrilotriacetic acid (Ni-NTA) resin (GE Healthcare). The insoluble material was reserved for later analysis via sodium dodecyl sulfate polyacrylamide
gel electrophoresis (SDS-PAGE). Protein was immobilized to the IMAC resin via batch mixing for 3 hours at 4°C. Following this incubation, the resin was batch washed with 30-40 bed volumes of wash buffer (20 mM phosphate pH 7.5, 150 mM NaCl, 25 mM imidazole). Protein was eluted from the column using wash buffer with imidazole at a final concentration of 200 mM. Formation of the triple helix was not spontaneous. IMAC purified protein was incubated for an additional 48-72 hours to allow for formation of the multimer. Size exclusion chromatography was performed using an Enrich SEC650 fast protein liquid chromatography (FPLC) column and BioRad Biologic System to separate the triple helix construct from the monomeric protein. Additional details regarding these processes can be found in previous work.\textsuperscript{14, 23}

**Gold Nanoparticle Synthesis and Preparation**

Citrate-modified AuNPs were first synthesized using a slightly modified citrate reduction protocol and the final NTA-modified AuNPs were obtained using a second ligand exchange method as described elsewhere.\textsuperscript{41-42} For the AuNP synthesis, 100 mM of H\textsubscript{3}AuCl\textsubscript{4}·3H\textsubscript{2}O stock solution and 200 mM of trisodium citrate (Na\textsubscript{3}C\textsubscript{6}H\textsubscript{5}O\textsubscript{7}) were prepared in deionized water. Next, 100 µL of stock H\textsubscript{3}AuCl\textsubscript{4}·3H\textsubscript{2}O was added to 50 mL of water and vigorously stirred for 1 min followed by the addition of 200 µL or 50 µL citrate stock solution for the creation of the 5 nm and 10 nm AuNPs, respectively. After 5 min of stirring, 100 µL of 100 mM NaBH\textsubscript{4}, freshly prepared in water, was added to the reaction solution and stirred for an additional 20 min for the reduction and formation of gold colloids. The 20 nm citrate-modified AuNPs were prepared in 50 µL of citrate stock solution in a similar manner except the solution was boiled for 30 min without NaBH\textsubscript{4}.\textsuperscript{41-42}
For preparing NTA-modified AuNPs, 10 mL of as-synthesized citrate-modified AuNPs were mixed with excess amount of mixed ligand stock solution (100, 50, 25 µL of 100 mM stock for the 5, 10, 20 nm AuNPs, respectively) containing 50% of dihydrolipoic acid (DHLA) and 50% NTA-appended dihydrolipoic acid (DHLA-NTA) which had been deprotected with an equivalent molar concentration of NaOH for an hour before being mixed with DHLA and the solution was stirred for 4 hours. DHLA-NTA was prepared as described.\textsuperscript{43} The final NTA-modified AuNPs were washed with water three times and purified using centrifugation with a centrifugal membrane filter (Millipore, 50-100K molecular weight cut-off membrane filter) to remove free unbound ligands. High resolution transmission electron microscopy (TEM) images were taken to confirm both the size and standard deviation of the AuNPs with a sample size of approximately $n = 100$ for each distinct AuNP size (5, 10, and 20 nm).

AuNPs were loaded with Ni$^{2+}$ for metal affinity coordination driven binding to the PTE$_3$’s terminal His$_6$ motifs using a cation exchange column procedure. Briefly, the cation exchange column (10 cm length × 1 cm diameter) was prepared using CM Ion Exchange Chromatography Resin (Biorad) and saturated with a 0.1 M NiCl$_2$ stock solution in water. The as-prepared NTA-modified AuNPs were added to the top of CM column and kept in the column for at least 30 min to promote the interaction between the Ni$^{2+}$ and NTA on the surface of the AuNP. The Ni$^{2+}$-NTA-modified AuNPs were eluted from the column using deionized water. The eluent solution was concentrated using a centrifugal membrane filter. For preparing PTE$_3$-AuNP, 25 µL PTE$_3$ of various molarities was allowed to self-assemble overnight with 25 µL of 5, 10, and 20 nm Ni$^{2+}$-NTA-modified AuNPs to complete the binding of the PTE$_3$ enzyme’s His$_6$-tag to the Ni$^{2+}$-NTA as schematically demonstrated in Figure 3.1. The enzyme to nanoparticle ratios were created by mixing the following molar concentrations for the 5, 10,
and 20 nm sized AuNPs. For the 5 nm AuNPs, 3 picomoles of AuNPs was mixed with 0 to 54 picomoles of PTE$_3$ to achieve PTE$_3$-AuNP ratios of 0 to 18 respectively while the final concentration of AuNPs in each sample was 120 nM. For the 10 nm AuNPs, 0.5 picomoles of AuNP was mixed with 0 to 5 picomoles of PTE$_3$ to achieve PTE$_3$-AuNP ratios of 0 to 10 PTE while the concentration of AuNP in each sample was 25 nM. For the 20 nm AuNPs, 0.25 picomoles of AuNPs was mixed with 0 to 2.5 picomoles of PTE$_3$ to achieve PTE$_3$-AuNP ratios of 0 to 10 while the final concentration of AuNPs in each sample was 5 nM.

**Transmission Electron Microscopy**

Structural characterization of as-prepared NPs was carried out using a JEOL 2200-FX analytical high-resolution transmission electron microscope (TEM) with a 200 kV accelerating voltage. Samples for TEM were prepared by spreading a drop (5~10 µl) of the filtered NPs dispersion (filtered using 0.25 µm Millipore syringe filters) onto ultrathin carbon/holey support film on a 300 mesh Au grid (Ted Pella, Inc.) and letting it dry. The concentration of NPs in the deionized water used was typically ~1-10 nM. Individual particle sizes were measured using a Gatan Digital Micrograph (Pleasanton, CA); average sizes along with standard deviations were extracted from analysis of ~100 nanoparticles of one sample.

**Dynamic Light Scattering**

Dynamic light scattering (DLS) measurements were carried out using ZetaSizer NanoSeries equipped with a HeNe laser source (λ = 633 nm) (Malvern Instruments Ltd, Worcestershire, UK) and analyzed using Dispersion Technology Software (DTS, Malvern Instruments Ltd, Worcestershire, UK). 10 nM concentration solutions of AuNPs, PTE$_3$-conjugated AuNPs or equivalent amount of PTE$_3$ were loaded into disposable cells and data were collected at 25°C. All the samples were prepared in 0.1X PBS buffer, pH 7.4. For each
sample, the autocorrelation function was the average of five runs of 10 seconds each and repeated between three to six times. CONTIN analysis was then used to quantify the hydrodynamic size profiles for the dispersions studied.

**Zeta-Potential**

For Zeta-Potential (ζ-potential) measurement, Laser Doppler Velocimetry (LDV) measurements were performed using a ZetaSizer NanoSeries equipped with a HeNe laser source (λ = 633 nm) (Malvern Instruments Ltd, Worcestershire, UK) and an avalanche photodiode for detection, controlled with DTS software. 10 nM concentration solutions of AuNPs, PTE₃-conjugated AuNPs or equivalent amount of PTE₃ were loaded into disposable cells and data were collected at 25°C. Three runs of the measurements were performed for each sample to achieve the zeta potential. All the samples were prepared in 0.1X PBS buffer pH 7.4.

**Confirmation of PTE₃-AuNP Self-Assembly**

AuNP-PTE₃ bioconjugates were confirmed by separation using agarose gel electrophoresis. AuNP-PTE₃ conjugates were loaded into wells of a 1% low electroendosmosis agarose gel supplemented with 1X TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3). A potential of 95 volts was then applied and the negatively-charged AuNP-PTE₃ conjugates were allowed to diffuse towards the anode. Images were taken ca. every 5 min on a Bio-Rad ChemiDoc Molecular Imager to visually monitor the differential changes in AuNP conjugate electrophoretic mobility in response to bioconjugation ratio.

**Enzyme Assays**

The insecticide paraoxon was used as a model OP substrate for the PTE₃ assays. PTE₃ hydrolyzes the phenol groups yielding a p-nitrophenol product which has a characteristic absorption centered at 405 nm (molecular extinction coefficient of ~18,000 M⁻¹cm⁻¹).
Experimentally, varying molar ratios of PTE$_3$ were functionalized to the 5, 10, and 20 nm AuNP. Two experimental formats were utilized. Fixed amounts of AuNPs where the molar ratio of PTE$_3$ per AuNP was systematically increased and fixed concentrations of PTE$_3$ where the amount of AuNPs was systematically decreased to increase the ratio of PTE$_3$/AuNP. Bioconjugate activity was assayed and compared to free PTE$_3$ using a similar method as previously described.\textsuperscript{44} It should be noted that the AuNP-PTE$_3$ ratios chosen were far below surface saturation in most cases to ensure that all enzymes were attached to the AuNPs. Conjugates and enzyme only controls were diluted into a final concentration series of paraoxon ranging from 20 to 5,000 µM within 384-well Corning flat bottom, non-binding microtiter plates.\textsuperscript{45} Assays were carried out in a Tecan Infinite M1000 dual monochromator multifunctional plate reader using a xenon flash lamp (Tecan, Research Triangle Park, NC) to measure the absorbance at 405 nm every 20 seconds. The absorbance values were converted to p-nitrophenol concentrations via a standard calibration curve of p-nitrophenol ranging from 0 to 200 µM.\textsuperscript{46} While there is a small overlap in extinction spectrum of colloidal AuNP and p-nitrophenol, the contributions of AuNP accounts for less than 0.1% of the total optical density due to the small concentration used (i.e., optical density originating from the 5, 10, and 20 nm AuNPs amounted to 0.002%, 0.013%, and 0.07% respectively as compared to the total optical density of each solution via UV-Vis spectrophotometer measurements). Therefore, the extinction spectrum of colloidal AuNPs is negligible as compared to the extinction spectrum of p-nitrophenol. All assays were performed twice and average values utilized with standard deviations where appropriate. Initial rates of PTE$_3$ activity and kinetic parameters were determined using the enzyme kinetics module within the SigmaPlot software which uses a non-linear curve fit for a single substrate.
Results and Discussion

PTE₃-AuNP Synthesis and Assembly

We have previously reported the enhancement of PTE and the PTE₃ trimer when attached to Cd-containing semiconductor QDs¹⁴,²⁰ and in this work the PTE₃ trimer is attached to a non-toxic, Cd-free AuNP. The PTE₃ trimer was prepared by appending the PTE gene to a collagen-derived triple helix domain and a trimerizing V domain followed by a terminal His₆ motif as described in the Methods. The PTE-collagen-His₆ monomer has an estimated Mw of ~53 kDa while the fully assembled PTE₃ trimer has an estimated Mw of ~155 kDa. The PTE₃ trimer was immobilized onto AuNPs of distinct diameter (i.e., 5 nm, 10 nm, and 20 nm) which were synthesized as described in the Methods. The native citrate stabilizing ligand was cap exchanged with a mixture of 50:50 DHLA and DHLA-NTA which provides the AuNPs with both aqueous colloidal stability across a broad pH and ionic range and the ability to spontaneously coordinate the PTE₃ protein via metal affinity coordination following charging of the NTA group with Ni²⁺ (see Figure 3.1).⁴⁷ This is, for all intents and purposes, the same interaction and mechanism by which the proteins were originally purified using commercial IMAC media. This approach allows for ratiometric control over the number of PTE₃ assembled to each AuNP simply by varying the molar amounts of each mixed together. It also allows for orientational control, i.e. displaying the active site away from the NP surface, a factor that is important for minimizing the conjugate’s structural heterogeneity which can, in turn, detrimentally decrease enzyme activity.⁴⁸ The PTE-His₆ motif, attached to the C-terminal of each of the three monomers, allows for the enzymatic binding pocket to orientate itself on the outward side of the enzyme-NP bioconjugate. Although each PTE₃ construct will ultimately display 3×His₆ on their termini due to its trimeric nature, functional results show no evidence
of crosslinking between the AuNPs, but rather excellent conjugate stability presumably due to the cumulative-synergistic nature of the multiple interactions at the NP interface.

Figure 3.1 **Left:** Schematic diagram of AuNP-PTE$_3$ conjugate preparation. AuNPs were surface functionalized with 50% dihydrolipoic acid (DHLA) and 50% nitrilotriacetic acid appended dihydrolipoic acid (DHLA-NTA). The NTA groups were preloaded with Ni$^{2+}$ as described in the Methods. PTE$_3$ coordinates by metal affinity to the Ni$^{2+}$-NTA displayed around the AuNPs to yield the final AuNP-PTE$_3$ bioconjugate. **Right:** Hydrolysis of paraoxon due to OPH catalyst into p-nitrophenol. Note, figure is not drawn to scale.

**AuNP and PTE$_3$-AuNP Bioconjugate Characterization**

To confirm the size of the as-synthesized AuNPs, high resolution transmission electron microscope (TEM) images were collected from each sample batch (Figure 3.2, Left). As confirmed in the representative TEM micrographs shown in Figure 3.2, the sample sizes (n~100) corresponded to 5 ± 0.9 nm, 10 ± 1.4 nm and 20 ± 3.0 nm diameter AuNPs. This, in turn, corresponds to approximate surface areas of ~79 nm$^2$, ~314 nm$^2$, and ~1,257 nm$^2$ along
with estimated curvatures of \( \sim 0.4 \text{ nm}^{-1} \), \( \sim 0.2 \text{ nm}^{-1} \), and \( \sim 0.05 \text{ nm}^{-1} \), respectively. Structural modeling, similar to that described previously,\(^{14,23}\) was used to estimate maximum enzyme packing densities of \( \text{ca.} 30, 50, \text{ and } 120 \) PTE\(_3\) that should fit onto the 5, 10, and 20 nm AuNPs. These large packing numbers result from the elongated PTE\(_3\) structure which places the large globular enzymes quite distal from the tight constraints of the NP surface where only the far smaller His\(_6\) motifs bind. This also assumes no steric hindrance between neighboring proteins and that the NTA binding moiety is equally displayed around the AuNPs in sufficient numbers to facilitate such a dense binding.\(^{49}\)

Agarose gel electrophoresis was next conducted to confirm PTE\(_3\) physically assembled to the AuNPs with ratiometric display (Figure 3.2, Right). Incrementally increasing ratios of PTE\(_3\)/AuNP were assembled to the NPs overnight as described in the Methods and loaded into 1\% agarose gels for electrophoretic separation. The gels were visualized at 5 minute intervals on a lightbox to monitor the effects of PTE\(_3\)-AuNP conjugation on migration properties. As shown in Figure 3.2, the unbound AuNPs migrate fastest towards the anode due to the high net negative charge from the DHLA and DHLA-NTA carboxyl end groups. As the ratio of assembled PTE\(_3\)-AuNP increases, the resulting migration of the complexes decreases in a manner that is directly proportional to the enzyme-AuNP ratio. This is a direct result of the increase in mass and hydrodynamic size along with changes to the overall net charge of the complex. The latter are quite complex to predict due to the different sizes and surface areas involved. The smaller 5 nm AuNPs displayed the largest change in migration as expected since their size is closest to that of the enzyme. Overall, this confirms that the PTE\(_3\) does assemble to these NPs with sufficient affinity to maintain the structure while being driven through a sieving matrix. Moreover, the assembly occurs in a direct ratiometric manner.
Figure 3.2 **Left:** Representative high-resolution transmission electron microscopy (TEM) micrographs of the 5, 10, and 20 nm AuNPs. **Right:** Agarose gel electrophoresis analysis of three different-sized AuNPs assembled with varying ratios of PTE₃ as indicated at the top. Images were collected every 5 min during separation.

DLS and zeta potential were also utilized to monitor the increase in hydrodynamic diameter (Hₐ) of the AuNP as a function of the increase in attached PTE₃. The hydrodynamic size and the zeta potential of the AuNPs were changed after conjugation of PTE₃, which confirmed that the conjugation was achieved after 2 hours of incubation. A Hₐ of 10.1 ± 0.65 nm, 17.1 ± 1.7 nm, and 50.5 ± 1.93 nm was measured for the initial 10 nm AuNP before functionalization, after DHLA/DHLA-NTA functionalization, and after conjugation with PTE₃ in a molar concentration ratio of 32:1 with the AuNPs, respectively. The measured hydrodynamic size of the PTE₃-conjugated AuNP was slightly larger than expected, which perhaps is due to the mild aggregation in the solution caused by excess amount of unbound PTE₃. The zeta-potential value of the 10nm AuNP for the same scenario decreased from -9.8 ± 1.72 for the AuNP (10 nm) before functionalization and to -28.7 ± 1.14, and -12.0 ± 1.5 mV after functionalization with DHLA/DHLA-NTA and PTE₃ (32:1 PTE₃ to AuNPs) respectively which suggests that the negatively charged surface of the AuNP becomes more charged with the DHLA/DHLA-NTA functionalization but less so with enzyme conjugation. The inclusion
of the enzyme increased the hydrodynamic drag (~17nm to ~50nm) which may have reduced
the mobility of the particle in the electrophoretic field neutralizing, to some degree, the zeta
potential.

**PTE₃-AuNP Activity Characterization via Fixed AuNP Concentration Method**

Full enzyme progress curves were first acquired by fixing the concentration of AuNPs
while *increasing* the bioconjugate molar ratio of PTE₃ displayed around the AuNP. This “fixed
AuNP concentration method” was carried out with 5, 10, and 20 nm AuNP scaffolds at 0.5 pM
(which corresponds to a gold mass concentration of 0.0008%, 0.006%, and 0.05%,
respectively)⁵⁰ while the PTE₃ concentration was varied to yield distinct AuNP-PTE₃ ratios
(Figure 3.3). The kinetic activity of the said PTE₃-AuNP bioconjugates was tested and
compared to an equivalent amount of control free PTE₃. The resulting changes in absorbance
from these experiments (See Figure A.1 and Figure A.2) were converted into product
concentration by linear interpolation to a *p*-nitrophenol calibration curve (Experimental
Methods). The initial rate of paraoxon hydrolysis for each of the PTE₃-AuNP conjugates and
corresponding controls of free enzyme were then plotted against the substrate concentration
and fitted with the Michaelis-Menten equation, see Figure 3.3a. The corresponding \(V_{\text{max}}\), \(k_{\text{cat}}\),
and \(K_M\) values for each of the assemblies and controls were also estimated from these data
(Figure 3.3b-d). Given the large concentration difference between AuNP conjugates and
substrate concentration, we note that in principle the experimental format meets standard
Michaelis-Menten and Briggs-Haldane assumptions.⁵¹

The ratios of PTE₃-AuNP utilized here were chosen so as to approach maximum
packing for the smaller AuNP and then subsaturation for the larger materials. As expected,
increasing PTE₃ concentration, both assembled on and off the AuNPs, increased the apparent

$V_{\text{max}}$ values, as more enzyme was able to hydrolyze more substrate (Figure 3.3b). For all the AuNP sizes and ratios tested, PTE$_3$ immobilization increased the observed $V_{\text{max}}$. At lower ratios of PTE$_3$-AuNP, the relative improvement in $V_{\text{max}}$ versus free enzyme was substantially higher displaying a nearly 17-fold increase at a 1:1 assembly ratio on the 20 nm AuNPs. Increases of 6.5-fold and 10.5-fold were noted for the 10 and 5 nm particles at the same ratio. On the other hand, when the ratio of PTE$_3$-AuNP increased to 24, $V_{\text{max}}$ only increased by approximately one third over the rate of free enzyme.

Comparing between the different size AuNPs, PTE$_3$ immobilization on the 20 nm AuNPs demonstrated an average enhancement (bioconjugate/free) of ~1.7x and ~1.1x higher than the same PTE$_3$-AuNP ratio of 10 and 5 nm, respectively. The catalytic rate ($k_{\text{cat}}$) of PTE$_3$ not attached to AuNPs was averaged in order to get a truer catalytic rate of free enzyme. As $k_{\text{cat}}$ ($V_{\text{max}}/[\text{PTE}_3]$) is directly correlated to $V_{\text{max}}$ there is an identical increase in $k_{\text{cat}}$ for PTE$_3$ attached to 5, 10, and 20 nm AuNP. As seen in Figure 3.3c, $k_{\text{cat}}$ for PTE$_3$-AuNP is roughly constant, irrelevant of the concentration of the enzyme for each sized AuNP with the average value of 11.0, 16.0, and 17.3 sec$^{-1}$ for 5, 10, and 20 nm, respectively, which is a ~1.7x (20/5 nm) and ~1.1x (20/10 nm) improvement. While $V_{\text{max}}$ and $k_{\text{cat}}$ show a significant improvement for PTE$_3$ immobilized on the AuNP, the Michaelis constant $K_M$, an indirect measure of enzyme-substrate affinity, remains similar to or higher than the free enzyme, suggesting that PTE$_3$ immobilization on the AuNP tends to decrease enzyme-substrate affinity. However, it should be noted here that the measured $K_M$ values for enzyme-NP conjugates are approximations at best since the enzyme-substrate complex cannot be strictly modeled as freely diffusing in solution. Moreover the Michaelis model overestimates the number of diffusing multivalent NP conjugates as well as underestimates the local concentration of
enzyme in the presence of the NP.\textsuperscript{19,20,25,34} We do note that similar increases in $K_M$ values have been noted for attaching other enzymes to the NPs.\textsuperscript{20} Finally, the catalytic efficiency of immobilized PTE\textsubscript{3} ($k_{cat}/K_M$) is decreased when on the 5 nm AuNPs, \textasciitilde0.70x, however, increased by \textasciitilde3.0x and \textasciitilde1.2x when immobilized on the 10 and 20 nm AuNPs respectively.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Representative PTE\textsubscript{3} enzymatic activity with fixed AuNP concentration. Experiments conducted with constant AuNP concentration while varying the PTE\textsubscript{3} ratio displayed on the 5 nm, 10 nm, and 20 nm AuNPs. (a) Initial rates for each of the increasing PTE\textsubscript{3} concentrations. Lines are fits to the data using the Michaelis-Menten formula while the symbols represent experimental data. Solid lines and squares are PTE\textsubscript{3} attached to the AuNPs.}
\end{figure}
while the dotted lines and triangles of the same color correspond to the identical concentration of PTE3 alone. Comparison of the (b) $V_{\text{max}}$, (c) $k_{\text{cat}}$ and (d) $K_M$ values derived from the data (a) across all enzymes ratios. Blue bars represent PTE3 attached to AuNPs and red bars represent equivalent PTE3 free in solution. Error bars are the standard error from estimating the indicated values using repeated data.

Interestingly, despite the smaller surface curvature of the larger AuNPs, which may impede enzyme conformational changes and increase the likelihood of detrimental enzyme neighbor interactions, enzyme activity appears to be most enhanced on the larger 20 nm AuNPs. This result is counter to some of our previous observations where smaller NPs manifested better enhancement of enzyme activity following attachment.$^{14, 20}$ However, this previous work primarily utilized direct enzyme attachment to the ZnS surface of CdSe/ZnS core/shell QDs while in this work both the NP constituents and bioconjugation chemistry are distinct. This suggests that attachment chemistry and NP material type plays a role in enhancing the enzymatic behavior for distinct enzyme-nanoparticle constructs.

**PTE3-AuNP Activity Characterization via Fixed PTE3 Concentration Method**

Full enzyme progress curves (See Figure A.3, Figure A.4, and Figure A.5) were next acquired by fixed concentration of PTE3 while increasing the bioconjugate molar ratio of PTE3-AuNP by decreasing the AuNP concentration. This “fixed PTE3 concentration method” was developed by fixing the PTE3 concentration at 1.0 pM, while the concentration of the 5, 10, and 20 nm AuNPs were varied. This assay was performed to validate and cross compare to the previous “fixed AuNP concentration method” assay and subsequently the same enzyme performance experiments were performed in this assay as was performed in the previous. The resultant initial rate of paraoxon hydrolysis by the PTE3-AuNP bioconjugates was plotted against paraoxon substrate concentrations (Figure 3.4a). Again, when PTE3 is displayed on to
the AuNP, most of the bioconjugates have improved initial velocities and this is also especially true for the bioconjugates at lower ratios of PTE₃/AuNP.

Figure 3.4 Representative PTE₃ enzymatic activity with fixed enzyme concentrations. Experiment conducted with constant PTE₃ concentration while varying the AuNP to PTE₃ ratio for the 5 nm, 10 nm, 20 nm AuNPs. (a) Initial rates with constant PTE₃ concentration as AuNP concentration decreased. Lines are fits to the data using the Michaelis-Menten formula while the symbols represent experimental data. Solid lines and squares are PTE₃ attached to the AuNPs while the dotted line and black triangles correspond to the identical concentration of PTE₃ alone. Comparison of the (b) $V_{\text{max}}$ (c) $k_{\text{cat}}$ and (d) $K_M$ values derived from the data in (a) across all the various ratios of enzymes used. Blue bars represent PTE₃ attached to AuNPs and red bars represent equivalent PTE₃ free in solution. The error bars are the standard error from estimating the indicated values using repeated data.
The corresponding $V_{\text{max}}$, $k_{\text{cat}}$, and $K_{M}$ values for each of the assemblies and control were also estimated from the fixed PTE3 concentration method assay and are compared as a function of ratio in Figure 3.4b-d. Figure 3.4b highlights the enhancements in PTE3 $V_{\text{max}}$ that were observed. In almost every case the incorporation of AuNPs increases $V_{\text{max}}$, with higher enhanced activity at lower ratios (1:1) and a slight decrease in activity at high ratios (1:32), 2.3x and 0.75x respectively for 20 nm AuNP. The same trends noted for $V_{\text{max}}$ values on the AuNP are also observed in the derived $k_{\text{cat}}$ values, Figure 3.4c. Although significantly higher in value than the free enzyme control, $k_{\text{cat}}$ appears to decrease as the ratio of PTE3-AuNP is increased. There is also an increase in relative $k_{\text{cat}}$ as the AuNP increases in size from 5, 10 and 20 nm. Similar to results found in the fixed AuNP concentration method, $K_{M}$ maintains a fairly consistent value for PTE3 immobilized onto the 5 and 10 nm AuNPs but increases (enzyme affinity worsens) for various PTE3 concentrations on the 20 nm AuNPs. Resultant enzyme efficiency ($k_{\text{cat}}/K_{M}$) calculations show an increase (5.0x) for the 5 nm AuNPs but a decrease on the 10 and 20 nm AuNP (0.68x and 0.67x respectively).

**Conclusions**

In this work, we demonstrated that PTE3 enzymatic activity can be significantly enhanced when immobilized onto AuNPs. Results demonstrated $V_{\text{max}}$ could be enhanced nearly 17-fold by immobilization on the 20 nm AuNP at low concentrations of PTE3 (5 pM) and low ratio of PTE3-AuNP (1:1). These results corroborate and exceed our previous results where PTE3 performance was enhanced when attached to multiple sized QDs—upwards of four-fold improvement in $k_{\text{cat}}$ for PTE3 bound to 525 nm QDs versus free floating in solution. Other researchers have explored the enhanced activity of enzymes immobilized to AuNPs and have observed similar results. For example, glucose oxidase immobilized on a 5 nm AuNPs
displayed increases an order of magnitude higher $V_{\text{max}}$ - 1.42 µM min$^{-1}$ mg$^{-1}$ immobilized on AuNP versus 0.25 µM min$^{-1}$ mg$^{-1}$ for free glucose oxidase.$^{38}$ Enhancement of other enzymes such as glycosylated beta-galactosidase, which is commonly used in lactose sensing, yielded over 10-fold increase in $V_{\text{max}}$ (soluble enzyme 0.58 µmol min$^{-1}$ mL$^{-1}$ and immobilized on AuNP 6.18 µmol min$^{-1}$ mL$^{-1}$).$^{52}$

Clearly, the number of PTE$_3$ attached to the AuNP affects the observed activity of the enzyme as loading more PTE$_3$ onto the AuNP decreases the relative magnitude of the enhancement. For example, PTE$_3$ displayed a significantly higher catalytic rate when immobilized at a 1:1 ratio of PTE$_3$/AuNP, but then showed a distinct decrease until the final 32:1 ratio (Figure 3.4). PTE$_3$ enzyme immobilized on the larger 20 nm also displayed the most significant increase in activity values with a $\sim$1.7x and $\sim$1.1x increase as compared to the same molar ratio of enzyme immobilized on the 5 and 10 nm diameter AuNP, respectively. The origins of these patterns are not easily dissected out of the current data. As mentioned, the observation of the largest enhancement in enzyme performance is noted where the lowest molar ratios of PTE$_3$ are immobilized on the nanoparticles—a behavior which mimics our previous reports on improving PTE$_3$ performance via immobilization on QDs.$^{14}$ Researchers postulate that the immobilization of enzymes in lower density on nanoparticles limits enzyme-to-enzyme neighbor interactions and hence increases the likelihood of proper enzyme conformation and subsequently higher enzyme activity or more specifically increases in the rate of enzyme-substrate to product conversion ($k_2$).$^{24, 20, 44}$ However, our results also demonstrate that PTE$_3$ activity is higher on larger sized nanoparticles (i.e., 20 nm vs. 10nm and 5nm) which we previously postulated may arise in part to the high localized density of
PTE$_3$ on the larger nanoparticles and overall increases in avidity of the enzyme-nanoparticle bioconjugate.$^{20}$

We do note a subtle but rather interesting discrepancy between the two experimental formats utilized here. In the first fixed AuNP concentration format, enzyme efficiency ($k_{cat}/K_M$) decreased on the 5 nm AuNPs and increased when the enzyme was immobilized on the larger NP materials. In the second fixed enzyme format, the converse of this pattern is observed. The origin of this pattern is not yet understood; however, we hypothesize that it is associated with enzyme packing and fitting on the NPs which could be far more constrained for smaller materials in the former experimental format. Moreover, investigation of beta-galactosidase activity when assembled on QDs also suggests the possibility of substrate accumulation or sequestration in the NP interface,$^{53}$ and this, too, would be constrained or limiting for smaller materials in the first experimental format by the increasing amount of enzyme present. Lastly, it is again worth pointing out the complexities of this interfacial environment and how much still remains unknown about it. Clearly, it is not unreasonable that subtle changes in experimental formats such as variations in surface chemistry; enzyme position/orientation and concentration; and substrate-to-nanoparticle attraction/diffusion rates could give rise to changes in the enzyme-nanoparticle structure and efficiency in which, in turn, changes the observed activity.$^{54}$

Clearly, far more work will be needed to elucidate the underlying mechanism(s) and nuances that give rise to the enhancement of enzymatic activity at a NP interface. These include surveying a far larger size range of NP materials, a range of NP surface chemistries, different types of enzymes and assays, and different types of NP bioconjugation chemistries. What these types of studies will ultimately provide, is insight into the nature of the NP-enzyme interface
and how this environment gives rise to enzymatic enhancement. Critically, the nanoscale interfacial environment, including the substrate and water boundary layers and gradients, immediately surrounding the enzyme-NP bioconjugate are postulated phenomena that gives rise to such enzymatic enhancement.\textsuperscript{23} Unfortunately, there are almost no metrologies currently available to probe and characterize this nanoscale interfacial environment,\textsuperscript{55} leaving the systematic and parametric functional assays suggested above as the more realistic but still indirect way forward.

Finally, the lower cost, facile fabrication protocol, and environmentally benign nature of AuNPs make them well suited as a scaffold that hosts and augments enzymes for rapid in-field OP sensors along with giving them excellent potential for incorporation into protective materials. A simple in-field OP sensor based on the PTE\textsubscript{3}-AuNP could be fabricated into a fluorescence sensor utilizing a smartphone in a single step readout.\textsuperscript{56} Similar photoluminescence sensors have been shown for point-of-care measurements of hydrolase activity in serum and whole blood using semiconductor QD FRET-based detection.\textsuperscript{7} Furthermore, this work adds to the ever growing body of NP-enzyme bioconjugates and suggests the application of NP-enzyme conjugates for medical, research, industrial and commercial utility which would certainly benefit from enhanced and accelerated enzymatic activity.\textsuperscript{25,57-58} Such enzyme-AuNP bioconjugates, and especially the phenomena that they exploit, could have a significantly broad impact on a variety of fields beyond biosensing to include environmental remediation, synthetic biology and even batch chemical processing.\textsuperscript{59}
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CHAPTER 4. HIGH RESOLUTION GRAPHENE FILMS FOR ELECTROCHEMICAL SENSING VIA INKJET MASKLESS LITHOGRAPHY

A Paper Published in ACS Nano

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Abstract

Solution-phase printing of nanomaterial-based graphene inks are rapidly gaining interest for fabrication of flexible electronics. However, scalable manufacturing techniques for high-resolution printed graphene circuits are still lacking. Here, we report a patterning technique [i.e., inkjet maskless lithography (IML)] to form high resolution, flexible, graphene films (line widths down to 20 µm) that significantly exceeds the current inkjet printing resolution of graphene (line widths ~ 60 µm). IML uses an inkjet printed polymer lacquer as a sacrificial pattern, viscous spin coated graphene, and a subsequent graphene lift-off to patterned films without the need for pre-fabricated stencils, templates or cleanroom technology (e.g., photolithography). Laser annealing is employed to increase conductivity on thermally sensitive, flexible substrates [polyethylene terephthalate (PET)]. Laser annealing and subsequent platinum nanoparticle deposition substantially increases the electroactive nature of graphene as illustrated by electrochemical hydrogen peroxide (H₂O₂) sensing [rapid response (5 sec), broad linear sensing range (0.1-550 µm), high sensitivity (0.21 µM/µA) and low detection limit (0.21 µM)]. Moreover, high-resolution, complex graphene circuits [i.e., an
interdigitated electrode (IDEs) with varying finger width and spacing] were created with IML and characterized via potassium chloride (KCl) electrochemical impedance spectroscopy (EIS). Results indicated that sensitivity directly correlates to electrode feature size as the IDE with the smallest finger width and spacing (50 µm and 50 µm) displayed the largest response to changes in KCl concentration (~21 kΩ). These results indicate that the developed IML patterning technique is well-suited for rapid, solution-phase graphene film prototyping on flexible substrates for numerous applications including electrochemical sensing.

**Introduction**

Solution-phase printing of nanomaterial-based conductive inks has helped facilitate the scalable manufacturing of flexible electronics\(^1\)\(^-\)\(^3\) in a low-cost, high-throughput fashion\(^4\)\(^-\)\(^6\). These printing protocols have expedited the advent of new technologies for diverse applications including those associated with energy storage,\(^7\) flexible electronic displays,\(^8\) smart packaging,\(^9\) and diagnostic sensors.\(^10\) Graphene-based inks have shown great promise in enabling these applications due to inherently advantageous material properties (e.g., high mechanical flexibility, electrical and thermal conductivity, chemical and environmental robustness, and biocompatibility).\(^11\)\(^-\)\(^13\) Numerous graphene printing techniques, such as screen, gravure, and inkjet printing have been developed to coat graphene flakes onto flexible and non-flexible surfaces.\(^14\)\(^-\)\(^15\) However, these techniques are often limited by low line resolution patterning (> 50 µms).

Recently, a variety of manufacturing tools have been developed to increase the line resolution of printed graphene films such as gravure templates and silicon stencils with line resolution of printed graphene films of 30 µm and 5 µm respectively.\(^16\)\(^-\)\(^17\) However, these techniques require the use of cleanroom technology (i.e., photolithography) to fabricate a
stencil or gravure template for each new pattern design. Photolithography requires multiple fabrication steps including photoresist application, development, and removal as well as UV exposure through a chrome/glass mask. This makes photolithography costly, time consuming, and inadequate for rapid prototyping of electrical circuits. Other groups have developed electrostatic spray deposition (ESD) to create interdigitated electrodes (IDEs) with finger width and spacing of 100 µm and 50 µm respectively. However, similar to previous techniques, this solution-phase graphene technique requires the need for photolithography patterning.

Inkjet printing is a scalable, cost effective, and versatile technique for depositing highly intricate patterns on multiple substrates. This process does not require fixed geometry masks/stencils or the need to use photolithography patterning. Moreover, inkjet printing has several advantages including: large surface area coverage, scalability for mass production, capability of printing on flexible substrates, and rapid prototyping through the use of computer-aided design (CAD) software. The major limitation of inkjet printing is the narrow value range that ink fluid properties (e.g., viscosity, surface tension, and density) must fall within for consistent droplet formation and pattern printing. For example, a typical inkjet printable ink must have a viscosity between 2-20 cP, surface tension between 30-40 mN/m, particle size less than 1% of the nozzle diameter, and a specific gravity of 1-1.5. With such stringent requirements, suspending large particle inks that can be printed with high resolution, without clogging nozzles, splattering, or inconsistencies in ink deposition, is technically challenging.

Herein we demonstrate a photolithography-free, high-resolution solution-phase graphene patterning technique, coined inkjet maskless lithography (IML). The IML technique can be used to pattern graphene films onto virtually any 2D planar substrate from rigid,
temperature resistant silicon to flexible, inflammable polymers. The method is accomplished by inkjet printing a polymer pattern, spin coating a more viscous/dense solution-phase graphene layer, and removing the polymer pattern via a solvent-based lift-off process to create the patterned graphene film. This inkjet printed patterning technique circumvents the need for developing templates and is conducive to scalable roll-to-roll manufacturing onto flexible substrates.\(^\text{21-22}\) Moreover, IML can be used to create graphene line resolutions of 20 µm—therefore superseding the typical resolution limitations of inkjet printing (width of ink droplet), which is typically greater than 60 µm.\(^\text{24}\) Some researchers have demonstrated inkjet printing polymers as a protective mask,\(^\text{25-27}\) while others have inkjet printed polymer layers for a sacrificial liftoff process as displayed in coffee-ring lithography\(^\text{28}\) and polymer microsieve pores.\(^\text{29}\) However, full patterning of high-resolution (< 25 µm) graphene circuits has not been previously addressed. Furthermore, we demonstrate the electrochemical utility of the developed graphene films by creating a hydrogen peroxide (H\(_2\)O\(_2\)) sensor printed on flexible Kemafoil® polymer substrate (heat treated PET). The patterned graphene was laser annealed and electrodeposited with platinum nanoparticles to increase electrode sensitivity. Finally, graphene IDE arrays with varying finger width and spacing (50 µm and 50 µm; 75 µm and 150 µm; 150 µm and 200 µm respectively) were manufactured and subsequently characterized with electrochemical impedance spectroscopy (EIS) to demonstrate the ability to create high resolution graphene circuits using IML.

Results and Discussion

Graphene Patterning via Inkjet Maskless Lithography (IML)

Overview of the IML Process Steps

The IML manufacturing protocol developed herein uses a four-step process to make conductive graphene patterns (Figure 4.1). First, the negative of the desired graphene pattern
is inkjet printed [designed with computer aided design software (CAD) and uploaded to the printer] onto the substrate with a sacrificial polymer (Figure 4.1a & Experimental Methods). Next graphene ink, made with a higher concentration of graphene than inkjet printable inks (e.g., 15 mg/mL vs ~3.5 mg/mL) is spin coated over the pattern (Figure 4.1b & Experimental Methods). Graphene adhesion is increased, and ink solvents are removed via heating (post-bake) in an oven (Figure 4.1c & Experimental Methods). Finally, the polymer pattern is removed and graphene lift-off occurs by exposing the substrate to a sonicated acetone bath (10 seconds) and/or direct acetone impingement with a wash bottle (Figure 4.1d).

**Figure 4.1** Schematic depicting the four-step manufacturing process for IML graphene patterning. (a) A sacrificial polymer layer is inkjet printed onto the substrate as the negative design pattern. (b) Highly concentrated graphene ink is spin coated evenly over the entire surface. (c) A post-bake process increases the adhesion of the graphene to the substrate. (d) The sacrificial layer is removed and graphene lift-off occurs with a sonicated acetone bath to produce the final graphene pattern.

**Sacrificial Polymer Ink Formulation and Printing**

The physical properties of the sacrificial polymer ink are critically important to create a high-resolution negative pattern. The fluid dynamic properties of the ink (viz., viscosity, surface tension, and density) strongly influences the inkjet printed line/pattern resolution. Primarily, two different non-dimensional properties [Reynolds number (Eq. 1) and Weber
number (Eq. 2) which are related to the inertial forces of viscosity and surface tension govern the printability of an ink,

\[
Re = \frac{v \rho a}{\eta} \\
We = \frac{v^2 \rho a}{\gamma} \\
Z = \frac{1}{Oh} = \frac{Re}{\sqrt{We}} = \frac{(\eta \rho a)^{1/2}}{n} \\
K_c = We^{0.5} \times Re^{0.25} \\
\]

where \( v \) is the impact velocity, \( \rho \) is the ink density, \( a \) is the drop diameter before impact, \( \eta \) is the viscosity of the ink, and \( \gamma \) is the surface tension. The Z-value (inverse of the Ohnesorge number, Oh), which describes the overall jettability of an ink (Eq. 3), combines Reynolds and Weber numbers and does not depend on the velocity of the jetted ink. For proper jetting to occur Reis and Derby et al., estimated the Z-value should be between 1 and 10 and the drop impact (Eq. 4) be below 100. At low Z-values (< 1) the viscosity of the ink is too large for proper ejection of the droplet, while at high Z-values (> 10) unwanted satellite droplets form. When the drop impact approaches 100, splashing upon impact is predicted, which decreases printing resolution. In this work, the sacrificial polymer ink was developed with the solvent cyclohexanone and terpineol, similar to previously reported jettable inks. These solvents were subsequently mixed with an acrylic lacquer at a ratio of 8:1:1, respectively (Experimental Methods). This ink displayed a Reynolds number of 30.8, Weber number of 26.9, and a Z-value of 5.9 when printed at 40° C which falls within the region of printable inks (Figure 4.2a). Hence, the developed polymer ink printed without satellite droplets, did not splash when deposited onto the substrate, and formed consistently stable drops upon expulsion from the piezoelectric nozzle of the inkjet printer (Figure 4.2b, blue arrows & Experimental Methods). The polymer printing process was adjusted (nozzle temperature set to 40° C, 20 µm
drop spacing) to develop well-defined printed lines (50-75 μm width), straight edges, and spacing between polymer layers below 25 μm (Experimental Methods). Upon impact, the inkjet printed polymer droplets coalesced into a film (Figure B.1).

Figure 4.2 Polymer lacquer fluid properties. (a) Graphical representation of optimized inkjet printing parameters plotted versus the non-dimensional Reynolds and Weber numbers. Star indicates where the developed printable polymer ink falls within these parameters. (b) Optical image of the polymer ink (~ dia. 10 μm) without any satellite droplets. The red arrow points to the 1 pL nozzle tips and the blue arrow indicates droplets acquired immediately after expulsion from the inkjet printer nozzle.

Graphene Spin Coating and Post-bake

Another important aspect for obtaining high-resolution graphene films is properly controlling the temperature and time of the graphene post-bake. Recall, that after the negative pattern is inkjet printed onto a substrate, a viscous graphene ink is spun over the polymer patterned surface (Figure 4.1 1 & Experimental Methods). A temperature and time controlled baking process is subsequently conducted in a convection oven to remove ink solvents and simultaneously improve the physical bond between the substrate and the graphene (Figure 4.3). At low baking temperatures and/or short baking times, the graphene did not adhere tightly to the substrate and was completely removed upon acetone lift-off of the sacrificial polymer (Figure 4.3, top left). At higher baking temperatures or longer baking times the polymer irreversibly hardened on the substrate which inhibited acetone removal of the
underlying sacrificial polymer (Figure 4.3, bottom right). However, a post-bake temperature and time of 120°C for 1 hr sufficiently adhered the graphene to the substrate while preventing over-hardening of the sacrificial polymer so that it could be removed by acetone; this post-bake time and temperature permitted the formation of well-defined graphene lines (25 µm width and 50 µm spacing) (Figure 4.3, center highlighted image).

Figure 4.3 A 5 × 5 panel of optical images illustrating the efficiency of the graphene lift-off method according to the temperature and time of the graphene post-bake. **Top Left:** Spin coated graphene and sacrificial polymer are completely removed exposing the bare Si/SiO₂ wafer (purple). **Bottom Right:** Spin coated graphene (green) completely covers the surface of the wafer as the underlying polymer sacrificial layer is not removed. **Center:** Highlighted center image shows high-resolution graphene lines with efficient graphene lift-off process. Scale bar equals 250 µm.
Graphene Annealing

The electrical conductivity of the graphene films was increased by laser or thermal annealing processes similar to our previous protocols where surfactants, solvents, and non-conductive binders (e.g., ethyl cellulose) are burned off at lower temperatures (< 300° C) or lower laser energy densities (< 50 mJ/cm²) and morphological changes (e.g., graphene flake fusion, superficial 3D nanostructuring or semi-vertical graphene petal formation) occurs at higher temperatures (> 800° C) or higher energy densities (> 70 mJ/cm²). The initial resistance of the IML patterned graphene before annealing was 135 ± 15 kΩ (n = 5) across a rectangular area of 25 mm x 3 mm. After thermal annealing at 1000° C for 60 min, the resistance of the sample (patterned on a Si/SiO₂ wafer) was reduced to 3.5 ± 0.25 kΩ (n=5) (Figure B.2). It is important to note that this annealing process was conducted in an inert ambient atmosphere, such as nitrogen, to ensure that the graphene did not oxidize at higher temperatures (> 350° C)—an effect that can subsequently hinder the electrical conductivity of the graphene. Alternatively, laser annealing was used to anneal the IML patterned graphene on temperature sensitive substrates (e.g., Kemafoil®, PET, polyimide) using a 1000 mW benchtop laser engraver. This laser annealing process (scan rate of 50 ms) reduced the graphene resistance to 329 ± 18 Ω (n = 5) (Figure B.2), which corresponds to a sheet resistance of ~90 Ω/sq and electrical conductivity of ~26,000 S/m.

Initially, the patterned graphene using the IML method is free of any oxygen functional groups. X-ray photoelectron spectroscopy (XPS) reveals distinct C-C bonds around 284 eV which is a combination of sp² (284 eV) and sp³ (284.8 eV) bonding structure, with no noticeable oxygen bonded to the graphene surface (Figure B.3a). Upon laser annealing in an oxygen atmosphere, an additional shoulder peak appears which represents oxygen groups (C-
O-C at ~286.5 eV and C=O at ~288.5 eV) functionalized to the surface of the graphene (Figure B.3b). As graphene oxide is relatively not conductive, and the graphene patterned using IML is conductive, the oxygen groups are most likely localized to the surface of the graphene where the heat/energy of the laser facilitates ambient oxygen groups binding to superficial graphene flakes.

**Printing Resolution**

The printed graphene lines were characterized with both confocal and atomic force microscopy (AFM) (Figure 4.4). Results indicate that removal of the sacrificial layer (Figure 4.4a) leaves a conductive graphene pattern with high-resolution down to 20 µm (Figure 4.4b), which is smaller than conventional inkjet printing techniques (~50-100 µm).\(^2\) Additionally, the IML process results in graphene lines with defined edges and relatively consistent height as opposed to inkjet printing which produces dome-like shaped cross-sections.\(^3\) Confocal microscopy revealed IML graphene printed lines with 20 µm width and 0.6 µm height (Figure 4.4c), along with sharp edges, and consistent spacing (Figure B.4). AFM was subsequently used (Figure 4.4d) to analyze surface morphology, which displayed a relatively even graphene deposition, but with high surface area due to randomly orientated superficial graphene flakes. AFM also revealed a 0.4 µm steep step at the edge of the patterned graphene with relatively smooth morphology across the surface of a 20 µm printed graphene line (Figure 4.4e – f). The difference between AFM and confocal microscopy height measurements is most likely due to different test locations of the spin coated graphene as spin coating deposits material more heavily in the center and becomes thinner as distance from the axis of rotation increases. Spin coating a second layer of graphene ink resulted in a doubling of the film thickness to 0.8 µm.
(see AFM measurements in Fig 4f, note that each height profile acquired at the same location on the sample).

Figure 4.4 Line resolution of graphene patterned with the IML method Si/SiO$_2$ wafer. (a) Optical microscopy images of inkjet printed polymer sacrificial layer, scale bar 100 µm. **Upper right corner:** Magnified inset shows 50 µm polymer lines with 20 µM spaces, scale bar 50 µm. (b) Graphene pattern after IML, scale bar 100 µm. **Upper right corner:** Magnified inset showing 20 µm graphene lines, scale bar 50 µm. Printed graphene lines analyzed with (c) confocal microscopy, (d) cross-sectional 3D AFM imaging, and (e) top view 2D AFM imaging. The dotted white line in (e) depicts the average cross section used to determine the graphene height profiles presented in (f). Averaged cross section height (f) of 1 (red) and 2 (blue) spin coated layers of graphene using IML.

**Electrochemical H$_2$O$_2$ Sensing with Graphene Films**

The electrochemical sensing capability of the patterned graphene using the IML method was first characterized via H$_2$O$_2$ sensing. A graphene electrode (25 mm x 3 mm) was fabricated using IML and laser annealing (Figure 4.5a). We have shown previously$^{30}$ that laser annealing fuses/welds together graphene boundary layers and significantly increases the printed graphene surface area by nano/microstructuring the orientation of superficial graphene flakes (Figure 4.5b). Laser annealing was used to increase the surface area and superficial defects which are well-suited for electrochemical, heterogeneous charge transport and metallic
nanoparticle deposition.\(^{34-35}\) Platinum nanoclusters (~25-50 nm diameter spheres) were subsequently electrodeposited onto the surface of the electrode, according to our previous protocols (Figure 4.5c, & Experimental Methods),\(^{35-36}\) as it is an efficient non-enzymatic catalyst for peroxide sensing. The laser annealed, high surface area graphene not only acts as a conductive transduction material, but also provides an effective scaffold structure for the platinum nanoclusters. We have shown in similar studies that this graphene/platinum hybrid dramatically improves \(\text{H}_2\text{O}_2\) sensing over platinum alone.\(^{35, 37-38}\)

![Figure 4.5](image_url)

**Figure 4.5** Electrochemical \(\text{H}_2\text{O}_2\) sensing characterization with the IML graphene electrode on heat treated PET. (a) Graphene electrode fabricated with IML, laser annealed, and electroplated with platinum nanoparticles to form an \(\text{H}_2\text{O}_2\) sensor. (b) Scanning electron micrograph (SEM) of IML graphene electrode before (top) and after (bottom) laser annealing. Scale bar 5 µm. (c) SEM of electrodeposited platinum nanoparticles onto graphene electrode. Scale bar is 5 µm. **Upper right corner:** Magnification of typical platinum nanoparticles on graphene. Scale bar equals 500 nm. (d) Cyclic voltammetry of \(\text{H}_2\text{O}_2\) sensor in 1x PBS (Black) with 100 µM \(\text{H}_2\text{O}_2\) additions. (e) Amperometric \(\text{H}_2\text{O}_2\) sensing showing current response for concentration step increases of 0.1 µm (orange arrows), 1 µm (purple arrows), 10 µm (green arrows) and 100 µM (red arrows) additions. Inset shows magnified view of 0.1 µM and 1 µM additions. (f) Concentration verses current graph illustrating the linear sensitivity of the \(\text{H}_2\text{O}_2\) sensor. Inset shows magnified view of current response for 0.1 µM, 1 µM, and 10 µM concentration additions.
To electrochemically characterize the platinum-graphene sensors, cyclic voltammetry and amperometry were with a standard 3-electrode set-up (Figure 4.5d – f). Cyclic voltammograms acquired with increasing concentrations of H₂O₂ (100 µM final concentration additions, from 0 to 500 µM) revealed that peak oxidation occurred at a voltage of approximately +0.4 V (Figure 4.5d). Subsequent amperometric measurements (Figure 4.5e-f) were conducted at a working potential of +0.4 V for increasing concentrations of H₂O₂ (i.e., 5 increments of 0.1 µM, 1 µM, 10 µM and 100 µM respectively). These amperometric results show a wide linear H₂O₂ sensing range (0.1 to 550 µM, \( R^2 = 0.996 \)), high sensitivity 0.21 µM/µA, low detection limit [0.21 ± 0.16 µM (3σ)], and a fast response time (~5 sec.). The H₂O₂ sensor compared favorably to those achieved by similar carbon/metal hybrids electrodes while eliminating the need for multiple processing steps (e.g., electrode polishing, drying under infrared lamps, multiple electrodepositions steps, and sonication cleaning) (Table 1).

Table 4.1 Performance comparison table of electrochemical H₂O₂ sensors comprised of carbon nanomaterial/metal nanoparticle hybrids.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Operating Potential (V)</th>
<th>Linear Range (µM)</th>
<th>Detection Limit (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtAu NC Graphene GCE</td>
<td>+0.1</td>
<td>0.82-8.73</td>
<td>0.008</td>
<td>39</td>
</tr>
<tr>
<td>PNEGHNS</td>
<td>0</td>
<td>1-500</td>
<td>0.008</td>
<td>40</td>
</tr>
<tr>
<td>OMCs/GE</td>
<td>+0.35</td>
<td>0.1-500</td>
<td>0.032</td>
<td>41</td>
</tr>
<tr>
<td>GNPs/GN-CS/GCE</td>
<td>-0.4</td>
<td>5-35000</td>
<td>1.6</td>
<td>42</td>
</tr>
<tr>
<td>CQDs/octahedral Cu₂O</td>
<td>-0.2</td>
<td>5-53000</td>
<td>2.38</td>
<td>38</td>
</tr>
<tr>
<td>CNF-PtNP/GCE</td>
<td>-0.34</td>
<td>10-9380</td>
<td>1.9</td>
<td>43</td>
</tr>
<tr>
<td>IML Pt-Graphene</td>
<td>+0.4</td>
<td>0.1-550</td>
<td>0.21</td>
<td>This Work</td>
</tr>
</tbody>
</table>

**PtAu NC Graphene GCE:** Platinum-gold nanoclusters on glassy carbon electrode. **PNEGHNS:** Platinum Nanoparticle Ensembled-on-graphene hybrid nanosheet. **OMCs/GE:** Ordered Mesoporous carbons modified glassy carbon electrode. **GNPs/GN-CS/GCE:** Gold Nanoparticle Graphene Chitosan modified glassy carbon electrode. **CQDs/octahedral Cu₂O:** carbon quantum dots octahedral cuprous oxide nanocomposites. **CNF-PtNP/GCE:** Nanoporous Carbon Nanofibers Decorated with Platinum Nanoparticles on Glassy Carbon Electrode. **IML Pt-Graphene:** Inkjet maskless lithography electrodeposited platinum on graphene.
Graphene Interdigitated Electrode (IDE) Film Fabrication and Characterization

To demonstrate the patterning and resolution capabilities of IML, complex graphene patterns and a high-resolution IML logo were developed and characterized. It should be noted here that such IML patterning is not limited to graphene inks but can also be conducted with other inks such as those derived from metallic nanoparticles (e.g., silver nanoparticles) (Figure B.5). An interdigitated electrode (IDE) array, a pattern consisting of two electrodes in alternating parallel bands with “comb-like features” that experiences large collection efficiencies, increased signal-to-noise ratios, fast response times, as well as no need for a separate reference electrode during electrochemical sensing, was next created with IML to test the electrochemical reactivity of such high-resolution graphene patterning. The IML graphene IDE was fabricated with finger widths of 50 µm and inter-finger spacing of 75 µm on silicon (Figure 4.6b & Figure B.6). Scanning electron micrograph (SEM) images of the graphene IDEs displayed well-defined graphene patterns with sharp edges, straight lines, and consistent widths (Figure 4.6b-c & Figure B.7). A high-resolution SEM corroborated well with the previous AFM measurements by displaying a sharp step height on the edge of the graphene pattern and a flat textured surface with high surface area due to the randomly orientated graphene flakes.

The graphene IDEs were electrochemically characterized by alternating current non-faradaic EIS in varying concentrations (10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ M) of potassium chloride (KCl). The direct relationship of impedance with frequency was analyzed via the Bode plot (Figure 4.6d). At lower frequencies (below 10 Hz), the impedance is related to the double layer capacitance, while at higher frequency (above 10,000 Hz) the dielectric region governs the impedance. The region in-between the double layer and the dielectric region is due to the
solution resistance in which changes in the concentration of ions and their mobility were analyzed. Subsequently, increasing the KCl concentration resulted in increased impedance in both the double layer and solution resistance regions.

Next, three distinctly sized graphene IDEs with two 10-finger combs of varying finger width and spacing (50 µm and 50 µm; 75 µm and 150 µm; 150 µm and 200 µm; finger width and spacing respectively) were created with IML. Resulting electrodes were characterized by EIS and the real verses imaginary impedance was plotted (Figure 4.6e). All graphene IDEs displayed typical Nyquist impedance characteristics: semicircle shapes with straight tails (45° straight line after semicircle) where the semicircular region (high frequency) is kinetically controlled and the tail region (low frequency) characterizes the mass-transfer controlled section (diffusion limited process). The double layer capacitance of the graphene IDE (50 µm finger width and 50 µm finger spacing) was calculated to be ~5 nF in 0.1 M KCl which is comparable to IDEs comprised of palladium, gold, and carbon nanotubes (Experimental Methods). As the concentration of KCl increased the equivalent film resistance increased making the system kinetically slower (wider semicircle) for each of the different feature sized graphene IDEs. As expected, the graphene IDE with larger feature sizes displayed larger film resistance ($R_{ct}$, diameter of semicircle). Additionally, the IDEs with smaller feature sizes demonstrated the most favorable sensing characteristics as they were more sensitive (change in diameter of semicircle, $\Delta R_{ct}$) to variations in the KCl concentration as impedance values recorded for IDEs with the smallest to largest feature sizes were 21 kΩ, 17 kΩ, and 4.5 kΩ respectively. These results demonstrate that the IDE fabricated using the IML method displayed standard EIS characteristics and hence can act as a viable EIS sensor; furthermore, decreasing the feature size of the electrochemical sensors can improve sensor sensitivity.
Figure 4.6 IML graphene design and IDE characterization. (a) Array of IDEs showing the negative IDE inkjet printed pattern using a sacrificial polymer layer (left) and the resultant graphene IDE pattern after IML on a silicon wafer (right). Upper corner images show magnified view of IDE patterns. (b) High resolution tilted-view SEM micrograph of resultant graphene IML IDE. Scale bar is 100 µm. (c) SEM displaying edge of graphene pattern. Scale bar equals 20 µm. (d) Diagram of total impedance vs. frequency for various concentrations of KCl (Bode Plot). (e) Nyquist impedance plots for three different size IDEs with four distinct concentrations of KCl.

Conclusions

In summary, a micro-manufacturing technique of depositing graphene films for high-resolution patterning has been demonstrated using a technique coined IML. This method can pattern solutions not easily inkjet printable such as inks comprised of high nanoparticle concentrations, large particle sizes, or higher viscosities. This developed IML process creates smaller feature sizes than conventional inkjet printing as the feature size is not limited to the width of the jetted material, but rather the space between two printed lines. In addition to high-resolution patterning, this process promotes rapid prototyping as no photolithography steps, stencils, or patterns are necessary.
Two different patterns were designed and tested to demonstrate the application of IML for electrochemical sensing. First, a H$_2$O$_2$ sensor was manufactured which showed the electroactive nature of the patterned and laser annealed graphene electrodeposited platinum hybrid using the IML technique. The designed H$_2$O$_2$ sensor exhibited a low detection limit (0.21±0.16 µM), wide linear sensing range (0.1 to 550 µM), and fast response time (5 sec)—the graphene films exhibited a high degree of electroactivity during electrochemical sensing. Next, IDEs of varying finger width and spacing (50 µm and 50 µm; 75 µm and 150 µm; 150 µm and 200 µm respectively) were manufactured to demonstrate the ability to create distinct high-resolution graphene circuits rapidly via the IML process. The patterned graphene IDEs had sharp edges, consistent line width, and demonstrated characteristic EIS measurements such as a double layer capacitance of ~5 nF. EIS measurements revealed that the sensor sensitivity correlated to electrode feature size as the IDE with the smallest finger width and spacing (50 µm and 50 µm) displayed the largest EIS magnitude response in KCl (~21 KΩ). The developed IML technique can be used to pattern solution-phase graphene on diverse substrates such as silicon as well as flexible, disposable substrates including Kemafoil® (heat treated PET), clear PET, and polyimide tape (Figure B.8). We have also demonstrated that other inks such as silver can be patterned with this IML technique (Figure B.5). In summary, this work shows great promise in providing a rapid prototyping method of high-resolution patterns for concentrated, conductive nanoparticle inks which is compatible with multiple substrates. Hence this technique could potentially have wide utility to applications that use patterned graphene including electrochemical sensors, energy harvesters, batteries, capacitors/supercapacitors, triboelectric nanogenerators, strain sensors, and chemical/biological sensors.\textsuperscript{43, 54-60}
Methods and Materials

Materials

Completely reduced graphene oxide (referred throughout as graphene) was purchased from ACS Materials (USA). All other chemicals including solvents were purchased from Sigma-Aldrich (USA), and used without any other further purification. The use of these chemicals is outlined in the following sections of this Experimental procedure section.

Polymer Lacquer Formulation and Printing

Polymer lacquer ink was designed and optimized using an acrylic lacquer and diluted with solvents to an inkjet printable range. In short, 1 mL of a toluene formaldehyde nail polish was mixed with 1 mL of terpineol and 8 mL of cyclohexanone. The solution was then vortexed for 1 minute and filtered using a 0.45 syringe filter. The viscosity and surface tension of the ink was measured using Rheometer uVisc micropipette viscometer at 40° C and were found to be 6 cP and 41 mN/m, respectively.

Inkjet printing of the polymer lacquer inks was conducted with a Fujifilm Dimatix Materials Printer (DMP2800). The polymer sacrificial ink was loaded into a 3 mL printer cartridge and printed through 1 pL nominal drop volume nozzles. The waveform was adjusted to print nozzles at a temperature of 40° C with a 20 µm drop spacing. These parameters yielded consistent droplets without any satellite droplets and well-defined lines that held tightly to the substrate. The ink was printed onto N-type <1,0,0> 300 nm dry thermal oxide polished silicon wafers (Silicon Quest International Inc.), polyimide (DuPont Kapton 125 µm), and heat stabilized polyester film (Coveme, Kemafoil® PET 100 µm). Typically, a single printed layer adequately forms the sacrificial layer, which dramatically increases the speed and resolution
of IML over typical inkjet printing, however porous materials required additional printing passes as the polymer was absorbed into the surface.

**Graphene Ink Formulation and Graphene Film Fabrication**

Graphene ink (10 mL) was synthesized by first vortexing 150 mg of completely reduced graphene oxide (ACS Material, GN1P0005, 1~5 µm flake size) in 50 mL of ethanol for 5 min at high speed in a 50 mL falcon vortex tube. Ethyl cellulose (viscosity 46 cP, 5% in toluene/ethanol 80:20(lit.), Sigma-Aldrich 433837) was added to the solution (25 mg/mL) and re-vortexed for 5 min on high to increase solution homogeneity. Next, 10 mL of terpineol (Sigma-Aldrich T3407) was added to the solution. The graphene ink was then poured into a 100 mL beaker and probe sonicated (Sonics Vibra-cell VCX-750 ultrasonic processor) at 70% amplitude with a 9 second pulse and 1 second rest for 2 hours to break up large particles. The beaker was suspended in a bath sonicator and the water was continually refreshed to provide cooling during sonication. The solution was then bath sonicated for 6 hours at high power to break up the graphene into smaller particles. The bath temperature was refreshed to maintain a temperature of no more than 40° C. The ethanol was then evaporated off by heating on a hotplate, leaving a concentrated graphene solution (15 mg/mL). This procedure provided a stable and homogenous graphene ink with a viscosity well-suited for spin coating (176.2 cP).

The graphene ink was spin coated over the entire surface of the substrate including the inkjet printed sacrificial layer. 1 mL of graphene ink was pipetted on the center of the wafer and spin coated at 1000 rpm for 30 seconds. The wafer was then heated at 90° C on a hotplate for 2 minutes to dry the graphene ink. A post-bake was performed in a convection furnace at 120° C for 1 hour to ensure the graphene thoroughly adhered to the substrate, or baked with a heat gun for 10 minutes. The sacrificial layer was then removed in an acetone bath and
impinged with acetone from a wash bottle or bath sonicated in a Branson 2800 series bath sonicator at low power for 10 seconds to remove excess graphene. It should be noted that adhesion between graphene flakes often created a thin graphene film layer that adhered to the edges of the patterned graphene and suspended over the sacrificial layer (Figure B.9a). Hence, this thin graphene film layer impedes the graphene lift-off process and prevented straight edge graphene lines. To circumvent these deleterious effects, the graphene electrodes were sonicated in an acetone bath for 10 seconds at low power or impinged with acetone from a wash bottle to remove excess graphene (Figure B.9b).

**Scanning Electron Microscopy (SEM)**

The IML patterned graphene was investigated using a Field Emission Scanning Electron Microscopy (FESEM) [FEI Quanta 250]. All images were captured using secondary electron (SE) mode, with a working distance of ~10 mm, spot size of 3.0, and with a 10 kV accelerating potential. Iridium was evenly coated using a turbo-pump sputter coater to deposit 2 nm of conductive coating over the samples to ensure no surface charging or capacitance.

**Atomic Force Microscopy (AFM)**

Atomic force microscopy images of printed graphene layers on silicon wafer surfaces were acquired in PeakForce Tapping mode using a Dimension Icon scanning probe microscope (Bruker, Santa Barbara, CA). The PeakForce Tapping images were acquired using ScanAsyst AFM probes used where model SCANASYST AIR (Bruker, Santa Barbara, CA). All images were acquired in air. AFM images were post processed using plane-fitting (second order) and/or flattening (zeroth order) techniques with Nanoscope software.
**Confocal Microscopy**

Confocal microscopy (Sensofar, S-neox, Spain) measurements were performed to study the surface features of printed graphene layers on prepared silicon wafers. An area of 350.88 × 264.19 μm² with 150 x magnification was scanned with the SensoScan software. The z-scan was performed using a 0.1 μm step size. Form removal post processing was performed to remove sample tilt.

**X-Ray Photoelectron Spectroscopy (XPS)**

X-ray photoelectron spectroscopy of IML patterned graphene before and after laser annealing was analyzed using a Kratos Amicus X-ray Photoelectron Spectrometer containing Al Kα excitation source (1486.7 eV). The binding energy scan was formed by subtracting the excitation energy from the measured photoelectron energy from the constituent elements. The C 1s spectrum fitted using CasaXPS with a Shirley background fitting and a Gaussian Lorentzian line peak fitting on the constituent peaks.

**H₂O₂ Sensor Fabrication and Electrochemical Characterization**

All electrochemical measurements and procedures were conducted on a CH instrument potentiostat (600E series) in 1x PBS buffer. The H₂O₂ sensors were fabricated using the IML method described herein. The electrodes were laser annealed at laser power 1 and raster rate of 50 milliseconds using a 1000 mW engraver with a blue-violet laser. Platinum was electrochemically deposited onto the graphene surface of the working electrode with a 2.5 mA/cm² current for 100 cycles following our similar established protocols for electrodepositing platinum nanoparticles on graphene and carbon nanotubes. The H₂O₂ sensors were next placed in a 3D printed container to standardize testing between electrodes; these sensors were tested using a standard three electrode setup with an Ag/AgCl
single membrane reference electrode and a platinum wire counter electrode (Figure B.10). Cyclic voltammograms were conducted with a scan rate 0.05 mV/s between the potential voltage of -0.2 to +0.6 V (Figure 4.5d). Amperometric voltammetry was conducted at +0.4 V as determined from cyclical voltammetry. Electrodes were polarized for approximately 250 seconds before H₂O₂ additions were added. H₂O₂ (Sigma Aldrich H1009) dilutions in 1x PBS was then added to create final concentration additions of 0.1 µM through 100 µM. The response time of the sensor was defined as the time from initial H₂O₂ injection to the point at which 95% of the steady state value was reached.

**Interdigitate Electrode (IDE) Fabrication and Electrochemical Characterization**

Graphene IDEs were manufactured using the IML method on a silicon wafer as described throughout the manuscript. The silicon wafer was then placed on a hotplate and thermally annealed at 300° C in ambient air for 30 min. A carbon paste was deposited on the ends of the IDE for alligator clips to attach. The IDE was tested in a standard two electrode setup by electrically shorting the reference and counter electrodes. Electrochemical impedance spectroscopy (EIS) was conducted with 5 mV amplitude, frequency between 1-10⁵ Hz, and no DC current bias. EIS was conducted in varying concentrations of KCl (10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ M). The double layer capacitance (Cₐdl) was calculated following the theory behind non-faradaic EIS which correlates Cₐdl with phase (ϕ), and impedance (|Z|) as a sampling function in the electrolyte solution. Cₐdl is hence calculated from the Nyquist plot by taking the inverse of the product of the film resistance (Rₖct, diameter of the semicircle) and angular frequency at top of the semicircle (ωₘₐₓZ⁺).
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CHAPTER 5. PRINTED GRAPHENE ELECTROCHEMICAL BIOSENSORS FABRICATED BY INKJET MASKLESS LITHOGRAPHY FOR RAPID AND SENSITIVE DETECTION OF ORGANOPHOSPHATES

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Abstract

Solution phase printing of graphene-based electrodes has recently become an attractive low-cost, scalable manufacturing technique to create in-field electrochemical biosensors. Here we report a graphene-based electrode developed \textit{via} Inkjet Maskless Lithography (IML) for the direct and rapid monitoring of triple-O linked phosphonate organophosphates (OPs); these constitute the active compounds found in chemical warfare agents and pesticides that exhibit acute toxicity as well as long-term pollution to soils and waterways. The IML printed graphene electrode is nano/microstructured with a 1000 mW benchtop laser engraver and electrochemically deposited platinum nanoparticles (dia. \textasciitilde 25 nm) to improve its electrical conductivity (sheet resistance decreased from \textasciitilde 10 000 \(\Omega/sq\). to 100 \(\Omega/sq\).), surface area, and electroactive nature for subsequent enzyme functionalization and biosensing. The enzyme phosphotriesterase (PTE), also known as organophosphate hydrolase (OPH), was conjugated to the electrode surface \textit{via} glutaraldehyde cross-linking. The resulting biosensor was able to rapidly measure (5 sec response time) the insecticide paraoxon (a model organophosphate) with a low detection limit (3 nM), and high sensitivity (370 nA/\(\mu\)M) with negligible
interference from similar nerve agents. Moreover, the biosensor exhibited high reusability (average of 0.3% decrease in sensitivity per sensing event), stability (90% anodic current signal retention over 1000 seconds), longevity (70% retained sensitivity after 8 weeks), and the ability to selectively sense OP in actual soil and water samples. Hence, this work presents a scalable printed graphene manufacturing technique that can be used to create OP biosensors that are suitable for in-field applications as well as, more generally, for low-cost biosensor test strips that could be incorporated into wearable or disposable sensing paradigms.

**Introduction**

Organophosphates (OPs) are widely used as insecticides to increase quality and yield of crops.\(^1\)\(^-\)\(^2\) While OPs are effective neurotoxins against several types of pests, they are also toxic to humans and the environment. High-level, acute exposure to OPs can lead to immediate detrimental health effects including miosis, rhinorrhea, apnea, convulsions and death.\(^3\) Subsequently, OP neurotoxins have even been weaponized into G-type chemical warfare agents such as Sarin and Soman,\(^4\)\(^-\)\(^5\) which were used in the 1994 Japan attack poisoning 600 residents\(^6\) and the 1999 Tokyo subway attack injuring 640 victims.\(^7\) In addition to such high-level acute exposure to OPs, chronic low-level exposure to OP-based pesticides is also a concern especially for farmworkers.\(^8\) For example, farmworkers in both developed and developing countries who work on fields sprayed with OPs expressed lower neurobehavioral performance than control farmworker groups.\(^9\)\(^-\)\(^10\) Despite their short half-lives (days to months), OP residues have been found in soils, sediments, and watersheds—raising health concerns for chronic and early-life exposure to non-occupational populations especially those living in agricultural communities.\(^11\)\(^-\)\(^12\) Hence rapid and accurate detection of even low-level concentrations of OPs in the environment are critical for effective remediation measures.\(^13\)
While there are numerous techniques capable of detecting trace levels of OPs (e.g., gas/liquid chromatography, immunoassays, and mass spectroscopy) these tests are time-consuming and must be performed in a laboratory by trained technicians.\textsuperscript{14-15} Currently, a rapid, inexpensive method of monitoring pesticide levels in water or soil slurries in the field does not exist.\textsuperscript{16}

Electrochemical, enzymatic biosensors presents a promising solution to in-field OP monitoring.\textsuperscript{17} Enzymatic biosensors exhibit favorable characteristics for point-of-service sensing (e.g., high selectivity, sensitivity, and reusability) for a wide variety of applications including medical screening,\textsuperscript{18} defense threat monitoring,\textsuperscript{19} and environmental testing.\textsuperscript{20} OP enzymatic biosensors have primarily focused on using cholinesterase enzymes (i.e., acetylcholinesterase or butyrylcholinesterase) that are inhibited in the presence of OP.\textsuperscript{21-22} While such inhibition-based enzyme biosensors have displayed high sensitivity and ultra-low OP detection limits, they also are prone to false-positive signals as cholinesterase enzymes can be inhibited by heavy metals or detergents that are found in soil and water samples.\textsuperscript{23} Comparatively, the enzyme phosphotriesterase (PTE (EC 3.1.8.1)) selectively binds to OPs via a three O-linked binding pocket that is selective to specific ester bonds found in many OPs such as paraoxon.\textsuperscript{24} PTE catalyzes paraoxon into equimoles of \( p \)-nitrophenol, an electroactive molecule, that can be readily monitored through direct oxidation at an applied potential of +0.95 V vs. a Ag/AgCl reference electrode.\textsuperscript{17} Electrochemical detection of paraoxon with carbon-based materials (e.g., glassy carbon, graphite and carbon nanotubes) have been used for the direct amperometric detection of \( p \)-nitrophenol, due in part to their high biocompatibility with immobilized enzymes and high electrical conductivity.\textsuperscript{25-30} However, due to low sensitivity (limit of detection \( \sim \)10 nM, Table 1) and the high cost associated with graphene and carbon nanotubes synthesized \textit{via} chemical vapor deposition, the potential of
PTE biosensors for direct, in-field sensing has been limited especially as the maximum allowable OP concentration levels in drinking water begins to approach the subnanomolar range (European Union Drinking Water Directive 98/83/EC).\textsuperscript{31} This manuscript addresses three key issues that often hinder enzymatic OP biosensors, \textit{i.e.}, scalable/economical manufacturing processes, surface-fouling, and low sensitivity/high detection limits. Herein, we report the first printed graphene biosensor for amperometric detection of the insecticide paraoxon, a model OP. Graphene electrodes were printed and patterned through a recently developed thin film manufacturing technique called Inkjet Maskless Lithography (IML).\textsuperscript{32} Next, the printed graphene electrodes (PGEs) were laser annealed and electrochemically deposited with platinum nanoparticles (PtNPs) to create a nano/microstructured surface that is highly conductive/electroactive with high surface area for increased enzyme loading and heterogeneous charge transport during electrochemical sensing. The graphene-based electrode was consequently biofunctionalized with the enzyme PTE through covalent crosslinking with glutaraldehyde. This biofunctionalized PtNP-IML-PGE demonstrated the lowest recorded detection limit (3 nM) and highest sensitivity (370 nA/µM) for any reported amperometric PTE biosensor to date. Moreover, the biosensor exhibited high reusability (average of 0.3% decrease in sensitivity per time sensed), stability (90% anodic current over 1000 seconds), longevity (70% retained sensitivity after 8 weeks), and selectivity (negligible interference to 6 similar nerve agents, ability to detect OP in water and soil samples). The performance and potential scalable manufacturing protocols of the PtNP-IML-PGE suggests they are well-suited for in-field pesticide detection or potential bioterror agent monitoring in actual biological matrices.
Methods and Materials

Chemicals and Reagents

Completely chemically reduced single layer graphene was purchased from ACS materials (GN1P005). All pesticides (including paraoxon) were obtained from Chem Service (N-12816). All other chemicals and solvents were purchased from Sigma-Aldrich and were used without any other purification unless otherwise stated. Water was filtered through a B-Pure Water Purification system (resistivity, 18.2 MΩ/cm²). Screen-printed carbon electrodes (SPCE) were purchased from CH Instruments (SE 101). River water was obtained from the Des Moines River in the state of Iowa.

Preparation and Purification of PTE

The gene encoding phosphotriesterase (EC 3.1.8.1), also known as organophosphate hydrolase (OPH), from Brevundimonas diminuta was synthesized by Genscript with flanking restriction enzyme sites that facilitated transfer to the bacterial expression vector pET28. Protein expression was performed in Escherichia coli strain BL21(DE3) (New England Biolabs. Ipswich, MA) as previously described.\(^{33}\) Briefly, cultures were grown in 500 mL shake flasks containing kanamycin (25 µg/mL) until mid-log stage. Expression of recombinant PTE was induced with 1 mM β-D-Isothiogalactopyranoside (IPTG) and continued overnight at 30 °C. Cell pellets were collected by centrifugation then lysed through a combination of EDTA treatment, lysozyme, and sonication. Insoluble material was pelleted via centrifugation and soluble proteins batched to immobilized metal affinity chromatography (IMAC) resin. Following an incubation to allow protein binding and batch washing to remove non-specifically bound proteins, PTE was eluted with 200 mM imidazole. The recombinant PTE was further purified by FPLC which also removed the imidazole. Protein concentration was
determined using a Nanosight 1000 to measure the absorbance at 280 nm. Calculations were based on the theoretical extinction coefficient and the molecular weight of the protein.

**Biosensor Fabrication**

**Printing Graphene via Inkjet Maskless Lithography (IML)**

Similar to our work published earlier, graphene electrodes were manufactured through Inkjet Maskless Lithography (IML)\(^{32}\). In summary, 150 mg of ethyl cellulose (viscosity 46 cP, 5% in toluene/ethanol 80:20), was completely dissolved in 50 mL of acetone and 10 mL of terpineol. Ethyl cellulose was used as a surfactant to increase the surface tension of the ink and subsequently promote graphene flake suspension in the ink and improve adhesion of graphene to the substrate upon printing. Pristine graphene flakes (150 mg) were added to the solution and were probe sonicated for 4 hours. Probe sonication was conducted at 70% amplitude with a 9 second pulse and 1 second rest cycle to reduce graphene size and increase graphene flake exfoliation. The ink was then filtered through a 0.8 µm syringe filter. Acetone was evaporated from the solution to yield a concentrated graphene ink (15 mg/mL) which was spin coated over an inkjet printed polymer sacrificial layer (10% formaldehyde resin, 80% cyclohexanone, 10% terpineol) on heat treated Polyethylene Terephthalate (PET (kemafoil, Coveme TSL W)). The substrate was then post-baked under a heat gun (Steinel #HB1750K) for 15 minutes at 1200 °F with a distance of 18 inches from the surface. The post-bake process significantly improved adhesion of graphene to the substrate without destroying the sacrificial polymer layer. The entire substrate was then impinged with an acetone wash-bottle to remove the sacrificial layer leaving highly defined electrodes.
Laser annealing

The IML graphene electrodes were laser annealed by a 1000 mW diode laser engraver (HTPOW). The wavelength of the laser was blue-violet (405 nm) which did not damage the heat-treated PET substrate. The carving time (i.e., burn time, is the amount of time the laser is positioned at one particular point on the substrate before moving onto a different substrate point) of the engraver was set at 20 ms. This carving time that was found to not degrade/destroy the integrity of the graphene electrode while maximizing its electrical conductivity and electroactive nature (Figure C.1 & Figure C.5).

Electrodeposited Platinum Nanoparticles

Platinum nanoparticles (PtNPs) were electrodeposited onto the surface of the electrode using a standard three electrical setup. A platinum wire (CH Instruments CHI115) was used as the counter electrode and a Ag/AgCl single membrane electrode was used as the reference electrode (CH Instruments CHI11). A platinum electroplating solution of chloroplatinic acid and sodium sulfate was made by mixing 4 mM of H$_2$PtCl$_6$ and 0.5 M Na$_2$SO$_4$. The electrode was then placed into the solution and PtNP were electrochemically deposited onto the surface by pulsed chronopotentiometry (istep) using pulses or cycles of 0.1 sec steps of 0 to 250 µA. This procedure provided high current density across the graphene surface which enhanced the nucleation of PtNP (25 ± 2.5 nm, n = 25) evenly across the graphene surface (Figure C.2). Diameter of nanoparticles was determined using SEM micrographs (Figure C.2) and ImageJ pixel measurement tool with a sample size of n = 25 PtNPs.

Phosphotriesterase Ink Preparation and Deposition

A PTE ink was developed by mixing 50 µL of PTE (15 µM with a molecular weight of ~35 kDa), 400 µL of DI water and 50 µL of 50 mg/mL Bovine Serum Albumin (BSA). The
PTE ink was thoroughly mixed using a desktop vortex (Fisher Vortex Mixer #02215365) on high for 15 seconds. BSA was added as it has shown to increase the stability of other enzymes and to a lesser extent to increase biosensor sensitivity. Glutaraldehyde (50 µL of 0.25%) was added to the solution to crosslink the enzyme to the surface of the electrode. The PTE ink was then drop coated onto the working electrode by hand-pipetting 10 µL directly onto the working electrode. The biosensors were then refrigerated (~4 °C) for at least 12 hours during enzyme incubation and were subsequently stored in this environment until biosensor testing.

**Electro-analytical Measurements**

All electrochemical measurements were conducted using a CH Instruments potentiostat (600E series) with a typical three electrode setup which included an Ag/AgCl reference and platinum wire counter. All tests were conducted in 15 mL of PBS Buffer (1x, Sigma Aldrich), modified to pH 8.0 with NaOH—a pH more favorable to PTE performance (Figure C.3). Cyclic voltammetry was performed with a 50 mV/s scan rate; slow scan rates were chosen in order to minimize surface charging effects due to the double layer capacitance emanating from the high surface area of the laser annealed graphene. To increase diffusion of substrate to the enzyme, a magnetic stir bar was added and rotated at 300 rpm for amperometric measurements. Paraoxon stock solution (1 mM) was made daily as it readily photo-hydrolyzes and was pipetted at various volumes for desired molarity for all amperometric measurements unless otherwise noted. For selectivity tests, diluted pesticide stock solutions of 0.1 mM were used as many of the tested interference pesticides have low solubility in water. Paraoxon calibration plots for the biofunctionalized PtNP-IML-PGE were conducted with a working potential of +0.95 V (vs. Ag/AgCl) as the biosensor detection limit was minimized at this working potential (Figure C.4). To remove the electromagnetic noise that was applied by the magnetic stirrer,
data was filtered by taking the running average of 100 points with a scan rate of 0.01 sec\(^{-1}\) (running average over 1 sec). Amperometric biosensor response time was calculated by monitoring the time from the release of target analyte from the pipette tip in the test vial to the time a new steady state current response (90% max anodic current) was recorded.

**SEM Images**

Scanning electron microscopy (SEM) images were acquired using a FEI Quanta 250 FE-SEM. All images were magnified to 15 000x using backscattering electrons, spot size of 4.0, and with a 10 kV potential. Iridium was evenly coated using turbo-pump sputter coater to deposit a 2 nm conductive coating over all samples to improve surface imaging by preventing electrons from tunneling though the graphene surface.

**Results and Discussion**

**Overview of the Biosensor Fabrication**

Paraoxon biosensors were fabricated by a simple four step process that utilizes printed graphene electrodes in lieu of graphene synthesized by chemical vapor deposition—a process requiring a high temperature (up to 1000°C) vacuum synthesis environment that is costly and not well-suited for scalable manufacturing (Figure 5.1 & Methods & Materials).\(^{37}\) The printed graphene electrodes used herein were first fabricated through our previously reported IML technique\(^{32}\) that includes inkjet printing a polymer mask, spin coating a graphene flake solution, thermal post-baking the printed graphene, and finally performing an acetone rinse lift-off to form well defined electrode patterns with a 5 mm diameter disk shaped working electrode (Figure 5.1a). After the graphene patterning process a laser diode engraver was used to anneal the graphene and remove non-conductive ink surfactants (Figure 5.1b). This laser annealing process significantly improves the electrical conductivity of the printed graphene
electrodes (sheet resistance decreases from \(\sim 10,000 \Omega/\text{sq.} \) to 100 \(\Omega/\text{s}\)) and adhesion of the graphene to the underlying PET surface while simultaneously nano/microstructuring the graphene surface which increases the electrode surface area and electroactivity (Figure 5.1b). Next, PtNP (diam. \(\sim 25 \text{ nm} \)) were decorated onto the laser annealed graphene through pulsed electrodeposition to further improve the electro reactivity of the biosensor (Figure 5.1c). Finally, PTE ink was drop coated onto the graphene platinum hybrid surface and consequently covalently bound to the surface via glutaraldehyde crosslinking (Figure 5.1d).

![Figure 5.1 Schematic diagram and corresponding SEM images depicting the fabrication process steps of PtNP-IML-PGE biosensor. (a) Graphene patterning via the IML technique, (b) laser annealing of IML graphene electrodes, (c) electrodeposition of PtNPs, and (d) drop coating enzyme (PTE) ink. Bottom: Surface SEM (15 000x) corresponding to each manufacturing step, scale bar signifies 2 \(\mu\text{m} \), inset (250 nm).](image)

**Laser Annealing Process**

The electrical conductivity of the Inkjet Maskless Lithography printed graphene electrode (IML-PGE) was significantly increased by a laser annealing technique (see Methods & Materials). The laser carving time (inversely proportional to raster rate) was incrementally increased from 5 ms to 20 ms which subsequently decreased the graphene sheet resistance two orders of magnitude from \(\sim 10,000 \Omega/\text{sq.} \) to 100 \(\Omega/\text{s} \) (Figure C.5) while increasing the porosity and micro/nanostructuring of the graphene surface (Figure 5.2a & Figure C.1) resulting in a
high electroactive surface (from no electroactive surface area to 53 mm$^2$ for non-annealed IML-PGE to 20 ms laser annealed IML-PGE, respectively, see Figure C.6). The electroactive surface area of the laser annealed IML-PGE was calculated using the Randles-Sevcik equation (Eq. 1).\textsuperscript{38-39}

$$i_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} v^{1/2} C$$  \hspace{2cm} (1)

where $n$ is the number of electrons in the faradaic reaction ($n = 1$), $A$ is the effective electroactive surface area (cm$^2$), $D$ is the diffusion coefficient (7.6 x 10$^{-6}$ cm$^2$/s), $C$ is the concentration of the bulk redox species (5 mM), $v$ is the scan rate (V/s), and $i_p$ is the current (A) at the oxidation peak acquired from the CVs in Figure C.6. Such increases in electrical conductivity and electroactive surface area significantly improve the oxidation rate of p-nitrophenol at the electrode surface as the sensitivity increases from 25 to 230 nA/µM (Figure 5.2b). However, further increases in laser carving time (i.e., 50 and 100 ms) begins to smooth the IML-PGE surface (electroactive surface area decreases from 53 mm$^2$ at 20 ms to 10 mm$^2$ at 100 ms, see Figure C.1 & Figure C.6) by enhancing inter flake bonding \textit{via} reforming of van der Waal bonds.\textsuperscript{40-41} Subsequently this graphene smoothing process significantly decreases the sensitivity of the electrode, three orders of magnitude from 230 nA/µM to 3 nA/µM, respectively. Hence the IML-PGEs used for all future experiments in this work are laser annealed with a carving time of 20 ms.

The laser annealing process most likely increased the electrical conductivity of the IML printed graphene due its ability to thermally degrade/destroy non-conductive surfactants and binders still present in the patterned graphene as well as to weld or sinter together individual printed graphene flakes\textsuperscript{32,40} Note this laser does not degrade the PET underlayer and can be tuned to not harm even paper-based substrates.\textsuperscript{40} Additionally, this laser annealing process,
performed in ambient air, increases the number of superficial defects while adding oxygenated species (COOH, -C=O, and -OH) to said defect as previously reported.\textsuperscript{42} The increase of graphene superficial defects has been shown to significantly increase the nucleation density of PtNP during electrodeposition.\textsuperscript{43} Such superficial oxygen species are well-suited for subsequent enzymatic biofunctionalization \textit{via} glutaraldehyde crosslinking as hydroxyl groups on the graphene surface bind to the aldehyde groups in glutaraldehyde.\textsuperscript{44} Moreover, this micro/nanostructuring also changed the surface wettability of the graphene from one that is hydrophilic (water contact angle (CA) < 10°) to one that is hydrophobic (CA ~90°) (insets Figure 5.2a).\textsuperscript{41} Such a hydrophobic electrode surface has been shown to repel the electrolyte sensing solution that consequently reduces the double layer capacitance and decreases the sensor background current; hydrophobic electrodes generally increase the signal-to-noise ratio of the output signal.\textsuperscript{45} Moreover, hydrophobic electrodes are generally less susceptible to surface-fouling as they tend to reduce the adsorption of non-specific species that may be endogenously found in biological matrices.\textsuperscript{46-48}

![Figure 5.2 SEM images portraying IML printed graphene (a) without and with laser annealing (20 ms carving time). Insets display corresponding water contact angle measurements. (b) Amperometric calibration plot of p-nitrophenol for the IML-PGE that has been laser annealed with distinct carving time (0-100 ms) (Left). Histogram of p-nitrophenol sensitivity vs. laser carving time (Right).](image-url)
**Platinum Nanoparticle Decoration**

PtNP (~25 nm in diameter) where electrodeposited onto the IML-PGE to further increase their electrocatalytic nature to \( p \)-nitrophenol oxidation (see Methods & Materials, Figure 5.3a).\(^{49}\) Initially the incorporation of PtNPs on the surface of the graphene improved the biosensor sensitivity as the number of deposition pulses or cycles (0 to 75 cycles corresponding to 195 and 275 nA/µM, respectively, Figure 5.3b). However, after 75 electrodeposition cycles, larger ridge-like macrostructures formed (Figure C.2) which significantly decreases the sensitivity (1000 cycles corresponding to 32 nA/µM, Figure 5.3b). This decrease in sensitivity is most likely due to the lower surface area and less catalytic capability of larger PtNP macrostructures as opposed to smaller PtNPs as previously illustrated with glucose biosensors fabricated with platinum nano/microparticles electrodeposited on CVD synthesized multi-layer graphene.\(^{43}\)

![Figure 5.3](image)

**Figure 5.3** Platinum deposition. (a) SEM micrographs displaying the decorating of PtNPs (diam. ~25 nm) on IML-PGE surface. Scalebar is 5 µm and the inset scalebar is 200 nm. (b) Paraoxon calibration plot for IML-PGEs with increasing electrodeposition cycles. Inset: Histogram depicting the sensitivity of the PtNP-IML-PGE to paraoxon.

Cyclic voltammetry (CV) was conducted in order to measure and compare the electrochemical sensitivity to \( p \)-nitrophenol with a screen-printed carbon electrode (SPCE) to that of an IML printed graphene electrode (IML-PGE) that has been laser annealed as well as
decorated with platinum nanoparticles (PtNP-IML-PGE) (Figure 5.4a & Figure C.6). All electrodes displayed distinct anodic peaks at an applied potential of +0.95 V vs. Ag/AgCl which corresponds to the oxidation of p-nitrophenol (1 mM). The IML-PGE and PtNP-IML-PGE both display a substantial increase in sensitivity to p-nitrophenol over the SPCE as faradaic oxidation peaks (at +0.95 V) were exhibited as follows: SPCE ~3 µA, IML-PGE ~10 µA, and PtNP-IML-PGE ~13 µA (Figure 5.4a). After the first scan cycle, the anodic current of the SPCE significantly decreased (>90%) due to surface-fouling (Figure 5.4a & Figure C.7). As p-nitrophenol oxidizes the phenol leaving group polymerizes on the surface of the electrode creating an insulating/diffusion barrier. Comparatively, both the IML-PGE and the PtNP-IML-PGE greatly resisted surface-fouling and decrease faradic current by less than 5%. We speculate that this resistance to surface-fouling is most likely due to the hydrophobic nature of the IML-PGEs as previously mentioned; similarly, other carbon nanomaterials have reported varying degrees of anti-fouling properties.50-52

The surface area of a SPCE and laser annealed IML-PGE and PtNP-IML-PGE were evaluated by performing CVs in ferri/ferrocyanide (Figure 5.4b & S6). The anodic and cathodic currents both increase in magnitude with faster scan rates and were plotted verses the square root of the scan rate. The linearity demonstrates the process is diffusion controlled; however, the faradaic peaks shift further apart—a characteristic that is commonly attributed to porous materials that experience slower target analyte diffusion rates.53 The Randles-Sevcik equation (Eq. 1 & Figure C.6) the electrochemically active surface area was estimated as 5, 53, and 56 mm² for SPCE, laser annealed IML-PGE, and laser annealed PtNP-IML-PGE, respectively.
Figure 5.4 Transduction layer characterization (a) Cyclic voltammetry showing 3 repetitive scans in 1 mM \( p \)-nitrophenol with SPCE (Green), laser annealed IML-PGE (Blue), and laser annealed PtNP-IML-PGE (Red). (b) Cyclic voltammetry of laser annealed PtNP-IML-PGE in 5 mM ferro/ferricyanide with various scan rates (5-50 mV/s). Inset: Randles-Sevcik plot: anodic and cathodic currents vs. square root of scan rate.

**Enzyme Characterization and Functionalization**

The enzyme PTE was converted into an ink and assayed to monitor and improve its performance. First, it should be noted that as paraoxon diffuse to the electrode surface, PTE catalyzes paraoxon into \( p \)-nitrophenol which is consequently oxidized at the PtNP-IML-PGE surface during electrochemical biosensing (Figure 5.5a). However, before electrochemical biosensing, absorption spectroscopy was used to verify the activity of the PTE that was expressed in *Escherichia coli* strain BL21(DE3) (See Methods & Materials). The enzymatic activity of the resultant PTE ink was fluorescently monitored in a plate reader assay. PTE degrades paraoxon by hydrolyzing the P-O bond yielding diethyl phosphate and \( p \)-nitrophenol which has a strong absorption at 405 nm with an extinction coefficient of \( \sim 18,000 \) \( M^{-1} \text{cm}^{-1} \), opposed to paraoxon which has minimal absorption (Figure 5.5a).\(^{54}\) Similar to methods
described, three varying ratios of PTE ink (0.5, 1, and 5% which accounts for 2, 4, and 20 nM PTE) were assayed versus varying concentrations of paraoxon (1, 2, 4, 8 µM). A standard enzymatic rate reaction model was constructed using Sigma Plot’s enzyme module (Figure 5.5b). As expected the higher concentration of PTE yielded higher $V_{\text{max}}$ as more substrate can hydrolyze more paraoxon, ~350, ~650, ~2300 nM/sec (0.5, 1, and 5%, respectively). All three ink concentrations yielded similar data for $k_{\text{cat}}$ (Turnover number), $K_M$ (Michaelis constant), and $k_{\text{cat}}/K_M$ (enzymatic efficiency) of ~160 sec$^{-1}$, ~0.270 µM, ~590 µM$^{-1}$sec$^{-1}$, respectively. These enzyme performance characteristics were a slight improvement compared to our previous reports for PTE synthesized in a similar fashion, and hence validated that the recombinant protein was properly expressed and incubated.

Figure 5.5 (a) Schematic diagram of PTE functionalized via glutaraldehyde to the PtNP-IML-PGE surface. Hydrolyses of paraoxon into $p$-nitrophenol due to immobilized PTE enzyme and subsequent oxidation of $p$-nitrophenol at the graphene electrode surface operating with a working potential of +0.95 V vs. Ag/AgCl. (b) Enzyme progress curve displaying the rate of $p$-nitrophenol production for various concentrations of paraoxon with enzyme inks created with distinct concentrations of PTE: 2 nM (Black), 4 nM (Green), 20 nM (Red).
The PtNP-IML-PGE was biofunctionalized with PTE enzymes by covalent crosslinking with glutaraldehyde and albumin. Glutaraldehyde crosslinking offers a straightforward and inexpensive approach to immobilizing enzymes onto electrode surfaces and in particular has shown to enhance the biosensor thermostability, reusability, and shelf-life when used to covalently link enzymes to graphene/graphene oxide electrodes.\textsuperscript{57-59} Distinct concentrations of glutaraldehyde (0.125, 0.175, 0.25, 0.325, 0.5\%) within a BSA/PTE mixture were tested to determine which improved the biosensor sensitivity the most (Figure C.8). Increasing concentration of glutaraldehyde from 0.125\% to 0.25\% significantly improved the biosensor sensitivity to paraoxon (most likely through increased loading of active enzyme on the electrode surface). However, the sensitivity begins to decrease after glutaraldehyde concentrations of 0.25\% due to an increased diffusion barrier which hinders paraoxon from binding to the enzyme and impedes p-nitrophenol from reaching the graphene transduction material after catalysis.

**Biosensor Performance Characterization**

The PTE biofunctionalized PtNP-IML-PGE was first calibrated in buffer solution via amperometry at an applied $+0.95$ V vs. Ag/AgCl (Figure 5.6a). The biosensor was capable of rapidly monitoring (response time $<10$ seconds) successive paraoxon concentration increases with distinct amperometric response signals for consecutive 0.1 $\mu$M paraoxon additions (blue arrows) and 1 $\mu$M concentration additions (red arrows). The biosensor displayed a semi-linear characteristic calibration plot that can be fitted with a second order polynomial ($i_{pa}=-0.011[\text{paraoxon}]^2 +0.350[\text{paraoxon}]$ where $i_{pa}$ is the peak anodic current response with a coefficient of determination ($R^2$) of 0.995. However, a linear fit of this calibration plot ($i_{pa} = 0.263[\text{paraoxon}]$) yielded a lower $R^2$ value (0.978). This deviation from linearity is most likely
due to the increased diffusion layer caused by the glutaraldehyde cross linking that limits the diffusion of paraoxon to the enzyme as well as the product (p-nitrophenol) to the electrode surface. Note that both the PtNP-IML-PGE and IML-PGE were biofunctionalized and linearly calibrated (Figure 5.6b); PtNP-IML-PGE displayed a higher sensitivity (370 nA/µM) and lower detection limit (3 nM, S/N=3) than the PTE biofunctionalized IML-PGE (270 nA/µM and 12 nM).

![Biosensor calibration plots](image)

Figure 5.6 Biosensor calibration plots. (a) Amperometric response of paraoxon additions: 0.1 µM additions (blue) and 1 µM additions (red) for the PTE functionalized PtNP-IML-PGE. Inset: corresponding concentration vs. current graph (second order polynomial fit). (b) Paraoxon concentration vs. amperometric current response for the IML-PGE (blue) and the PtNP-IML-PGE (red) (linear fit). Error bars are standard deviation of 3 runs (n = 3).
These biosensor results compare quite favorably with other paraoxon electrochemical biosensors. For example, the PtNP-IML-PGE biosensor displayed the highest sensitivity and lowest detection limit of any reported electrochemical biosensor that utilizes the PTE enzyme to detect paraoxon (Table 1). Note that the PtNP-IML-PGE biosensor paraoxon sensitivity, detection limit, and linear range reported in Table 1 were all calculated using the linear fit to the calibration plot to be congruent with standard reporting practices (Figure 5.6b). The developed biosensor could also be implemented into high-volume manufacturing protocols (e.g., roll-to-roll polymer printing and graphene coating) as there is no need for photolithography steps and electrode patterning can be conducted on flexible, disposable substrates. Additionally, the biosensor design eliminated the need for multiple processing and sensing steps (e.g., electrode polishing, multiple electrodeposition steps, a faraday cage, flow injection, or ion exchange membranes) that have been reported with previous paraoxon biosensors (Table 1).

Table 5.1 Performance of various direct PTE amperometric biosensors

<table>
<thead>
<tr>
<th>Material/Immobilize</th>
<th>Sensitivity (nA/µM)</th>
<th>Detection Limit (nM)</th>
<th>Linear Range (µM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-SWNT/EDC-NHS</td>
<td>2.4</td>
<td>10</td>
<td>0.5-8.5</td>
<td>50</td>
</tr>
<tr>
<td>GC-CLEC/GA</td>
<td>25.95</td>
<td>314</td>
<td>0.5-2.0</td>
<td>34</td>
</tr>
<tr>
<td>GC-MWNT/Na - FI</td>
<td>25</td>
<td>150</td>
<td>0.25-4</td>
<td>51</td>
</tr>
<tr>
<td>FI-Au-Ny/GA</td>
<td>2.29</td>
<td>100</td>
<td>1-10</td>
<td>60</td>
</tr>
<tr>
<td>MWNT-DNA/PEI</td>
<td>74</td>
<td>77</td>
<td>-</td>
<td>61</td>
</tr>
<tr>
<td>C-Ny/GA-Si</td>
<td>12</td>
<td>20</td>
<td>0.02-18</td>
<td>28</td>
</tr>
<tr>
<td>GC-MC-CB/Na</td>
<td>198</td>
<td>120</td>
<td>0.2-8</td>
<td>25</td>
</tr>
<tr>
<td>C/Na</td>
<td>1.45</td>
<td>400</td>
<td>4.6-46</td>
<td>62</td>
</tr>
<tr>
<td>GC-MC/Na</td>
<td>129</td>
<td>9</td>
<td>0.5-25</td>
<td>52</td>
</tr>
<tr>
<td>IML-PGE/GA</td>
<td>270</td>
<td>12</td>
<td>0.1-1</td>
<td>This Work</td>
</tr>
<tr>
<td>PtNP-IML-PGE/GA</td>
<td>370</td>
<td>3</td>
<td>0.1-1</td>
<td>This Work</td>
</tr>
</tbody>
</table>

GC – Glassy Carbon; SWNT – Single-walled Carbon Nanotubes; CLEC – Cross-linked Enzyme Crystals; GA – Glutaraldehyde; MWNT – Multi-walled Carbon Nanotubes; Na – Nafion; FI – Flow Injection; PEI – Polyethyleneimine; C – Carbon; Ny – Nylon; Si – Silicon Oil; MC – Mesoporous Carbon; CB – Carbon Black; PtNP – Platinum Nanoparticles; IML – Inkjet Maskless Lithography; PGE – Patterned Graphene Electrodes

The PTE biofunctionalized PtNP-IML-PGE also demonstrated a high degree of stability, reusability, and selectivity. For example, the biosensor retains a steady (90%) anodic
current over 1000 sec after 1 µM paraoxon concentration additions, demonstrating little surface polymerization and good operational stability (Figure 5.7a). Such resistance to surface fouling may be due to the hydrophobic nature of the laser annealed IML-PGE, as previously mentioned. Long-term biosensor stability was evaluated by testing the biosensors each week for 8 weeks (Note the biosensors were stored at 4°C when testing was not occurring.). The biosensor retained nearly 70% and 50% of initial sensitivity to paraoxon for PtNP-IML-PGE and IML-PGE, respectively (Figure 5.7b). This retainment in enzyme activity could be attributed to the biocompatibility of the graphene as well as the strong crosslinking of glutaraldehyde, all of which may prevent enzyme denaturation. The biosensor also demonstrated a strong degree of reusability and retained 95% sensitivity to paraoxon even after 12 repeated uses (average of 0.3% decrease per time sensed, Figure 5.7c).

Figure 5.7 PtNP-IML-PGE biosensor characterization. (a) Stability test: steady oxidation current of 1 µM paraoxon addition over 1000 sec. (b) Longevity test: sensitivity of biosensors tested over 1-week intervals. (c) Reusability test: sensitivity of a single biosensor with repeated test runs. (d) Selectivity test: 5 µM spikes of paraoxon (red), p-nitrophenol (green), chlorpyrifos methyl (blue), Parathion (yellow), dichlofenthion (purple), fenitrothion (orange), phoxim (grey), and dimethoate (pink).
Next, the selectivity of the designed biosensor was evaluated against potentially interfering nerve agents and within actual soil slurries. It should be noted here that OP sensors that rely on PTE are selective as the enzyme specifically targets triple O-linked phosphonate centers such as found in paraoxon, parathion, chlorpyrifos and chemical warfare agents such as Sarin and Soman. The selectivity of the biosensor was analyzed during amperometry by adding OP concentrations (5 µM additions) into buffer solution along with various pesticides. The biosensor exhibited a large current increase for paraoxon and $p$-nitrophenol, but an undetectable amperometric increases for methyl parathion and chlorpyrifos (Figure 5.7d). The biosensors inability to detect these ubiquitous phosphates is consistent with the reported class of PTE that catalyzes these pesticides at a much slower rate. Additional pesticides that do not contain phenolic leaving groups or are not catalyzed by PTE (dichlofenthion, fenitrothion, phoxim, and dimethoate) were also tested and do not show any substantial increase in oxidation current. Paraoxon was then added again to the solution and a similar step height was observed demonstrating that none of the pesticide competitively inhibited PTE. The biosensor was next tested in a variety of biological solutions (tap water, river water, and a soil slurry) to evaluate its selectivity within actual biological matrices. All solutions were filtered with a 0.45 µm syringe filter, pH adjusted to 8.0 with NaOH, and ionic strength increased to 0.01 M PBS. The biosensor was able to detect low levels (5 µM) of paraoxon in a variety of real world solution mediums (Table 2). All biosensor test results were within 10% of pure buffer solutions (based on second order polynomial calibration plot, Figure 5.6a) demonstrating the biosensor is amendable to in-field or point-of-service testing in water and soil samples.
In conclusion, an amperometric biosensor for the detection of OPs (paraoxon) has been developed based on crosslinked PTE onto printed graphene electrodes that has been nano/microstructured via laser annealing and PtNP electrodeposition. Graphene provides an effective transduction material for rapidly monitoring the oxidation of $p$-nitrophenol due to high conductivity, heterogenous charge transport, and biocompatibility. The transduction layer was further enhanced through laser annealing which fused graphene flakes together, increased surface area, and provided stable attachment locations through oxygen functional groups. The high surface area creates a hydrophobic biosensor that resists surface fouling by repelling non-specific species from adsorbing on the electrode surface. PtNP were utilized to increase sensitivity and improve biosensor performance (increased surface area and improved biosensor stability). The resulting biosensor exhibited the lowest detection limit (3 nM) and highest sensitivity (370 nA/µM) of any electrochemical PTE biosensor. The designed biosensor displayed a stable response to paraoxon (retained 90% anodic current over 1000 sec), long-term stability (70% over 8 weeks), reusability (90% after 12 repeated scans), and high selectivity to paraoxon. Finally, the biosensors were tested in real samples (tap water, river water, and a soil slurry) to demonstrate in-field biological matrices.

Inkjet Maskless Lithography (IML) provides a rapid and low-cost process to manufacture highly defined graphene electrodes for electrochemical sensing. PtNP-IML-PGE also can be implemented into a high-volume manufacturing (e.g., roll-to-roll processing) as it

<table>
<thead>
<tr>
<th>Solution</th>
<th>Addition Spiked (µM)</th>
<th>Concentration Detected (µM)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap Water</td>
<td>5</td>
<td>5.35</td>
<td>107</td>
</tr>
<tr>
<td>River Water</td>
<td>5</td>
<td>5.10</td>
<td>102</td>
</tr>
<tr>
<td>Soil Slurry</td>
<td>5</td>
<td>4.75</td>
<td>95</td>
</tr>
</tbody>
</table>

Conclusions
does not require high cost photolithography steps and is patterned on a low-cost, disposable substrate. The designed biosensor utilizes PTE that is selective to triple O-linked phosphonate centers; therefore, it is advantageous over cholinesterase biosensors which require additional substrates, incubation periods, and are not selective to only organophosphates. In-field application of this biosensor could present farmers with a rapid, point-of-application sensor to monitor pesticide levels, which allows for a more accurate application of OPs, decreasing negative environmental impacts. The biosensor could be employed for drinking water testing\textsuperscript{63-64} or defense threat monitoring,\textsuperscript{20,65} via a Boolean enzyme cascade for example, to provide high fidelity warnings for proactive measures to be taken to protect human life.\textsuperscript{66} Additionally, since the printing, laser annealing, and functionalization protocols can all be performed on thermally and chemically sensitive materials (e.g., polymers and paper), the developed graphene-based OP biosensor could be incorporated into wearable ring-based, glove-based, or textile-based sensor platforms for rapid in-field analysis and defense threat awareness of nerve agents.\textsuperscript{67} Finally, the PtNP-IML-PGE fabrication protocol could be used for the scalable manufacturing of printed graphene-based electrodes for other applications in addition to biosensors including high-performance supercapacitors,\textsuperscript{68} biofuel cells,\textsuperscript{69} as well as dye-sensitized solar cells.\textsuperscript{70}

References


CHAPTER 6. ENHANCED ELECTROCHEMICAL BIOSENSOR AND SUPERCAPACITOR WITH 3D POROUS ARCHITECTURED GRAPHENE VIA SALT IMPREGNATED INKJET MASKLESS LITHOGRAPHY

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Abstract

Advances in solution-phase graphene patterning has provided a facile route for rapid, low-cost and scalable manufacturing of electrochemical devices, even on flexible substrates. While graphene possesses advantageous electrochemical properties of high surface area and fast heterogenous charge transport, these properties are attributed to the edge planes and defects sites, not the basal plane. Herein, we demonstrate enhancement of the electroactive nature of patterned solution-phase graphene by increasing the porosity and edge planes through the construction of a multidimensional architecture via salt impregnated inkjet maskless lithography (SIIML) and CO\textsubscript{2} laser annealing. Various sized macroscale pores (<25 to ~250 µm) are patterned directly in the graphene surface by incorporating porogens (\textit{i.e.}, salt crystals) in the graphene ink which act as hard templates for pore formation and are later dissolved in water. Subsequently, microsized pores (~100 nm to 2 µm in width) with edge plane defects are etched in the graphene lattice structure by laser annealing with a CO\textsubscript{2} laser, simultaneously improving electrical conductivity by nearly three orders of magnitude (sheet resistance decreases from >10 000 to ~50 Ω/sq.). We demonstrate that this multidimensional porous graphene fabrication method can improve electrochemical device performance through design.
and manufacture of an electrochemical organophosphate biosensor that uses the enzyme acetylcholinesterase for detection. This pesticide biosensor exhibits enhanced sensitivity to acetylthiocholine compared to graphene without macropores (28.3 µA/nM to 13.3 µA/nM) and when inhibited by organophosphate pesticides (paraoxon) has a wide linear range (10 nM to 500 nM), low limit of detection (0.6 nM), and high sensitivity (12.4 nA/nM). Moreover, this fabrication method is capable of patterning complex geometries [i.e. interdigitated electrodes (IDEs)] even on flexible surfaces as demonstrated by an IDE supercapacitor made of SIIML graphene on a heat sensitive polymer substrate. The supercapacitor demonstrates a high energy density of 0.25 mWh/cm$^3$ at a power density of 0.3 W/cm$^3$. These electrochemical devices demonstrate the benefit of using SIIML and CO$_2$ laser annealing for patterning graphene electrodes with a multidimensional porous surface even on flexible substrates and is therefore a platform technology which could be applied to a variety of different biosensors and other electrochemical devices.

**Introduction**

Graphene has emerged as one of the most attractive electrocatalytic transduction materials due to its extraordinary electrical and thermal conductivity, high mechanical strength, biocompatibility, high carrier capacity/mobility, and potential high surface area.$^{1-3}$ Specifically, electrodes comprised of graphene and graphene derivatives (graphene nanoparticles, reduced graphene oxides, oxidized graphene, functionalized graphene) have demonstrated high electrical conductivity and/or catalytic capability. For example, in the past decade graphene has been used for electrode materials in electrochemical devices such as supercapacitors,$^4$ batteries,$^5$ fuel cells,$^6$ cell electrode stimuli$^7$-$^8$ and sensors.$^9$-$^{10}$
Graphene is typically produced by chemical vapor deposition (CVD) which can yield nearly pristine (defect-free) single or few layer 2D sheets.\textsuperscript{11} In the context of electrochemistry, these 2D pristine sheets or basal plane geometry exhibit four order of magnitude lower specific capacitance and seven orders of magnitude lower electron transfer rates than the edges planes or edge defect sites of the graphene.\textsuperscript{12} Numerous methods have been used to increase the electrochemical surface area (ECSA) of graphene. For example, nanosphere lithography using self-assembly of polystyrene,\textsuperscript{13} silica,\textsuperscript{14} or MnO\textsubscript{2}\textsuperscript{15} nanospheres has been shown to etch nanopores into CVD grown graphene to increase the defect density in the lattice structure (more edge planes) and consequently improved the sensitivity of electrochemical sensors. Others have constructed three-dimensional CVD grown graphene foam out of a nickel scaffold\textsuperscript{16}, polystyrene colloidal particles as a sacrificial template,\textsuperscript{17} as well as various other manufacturing methods derived from chemical vapor deposition, hydrothermal methods, and sugar-blowing production.\textsuperscript{18} While these methods significantly increase the ECSA of the graphene electrode, they often require clean room technology, and/or high temperature fabrication. Such complex fabrication protocols are hence expensive and not amenable to high-throughput manufacturing such as roll-to-roll fabrication.

Solution-phase graphene provides an attractive alternative to costly CVD grown graphene as it can be synthesized from low-cost graphite with mechanical/chemical exfoliation.\textsuperscript{19-20} Various methods have been exploited to increase the ECSA of solution-phase graphene. For example, the “breath-figure” method uses moist gas that condenses on organic solution-phase graphene which results in the formation of highly controlled evaporated water droplet pores (~2 µm).\textsuperscript{21-22} Others have electrochemically reduced graphene oxide which provides a simple, one-step procedure to create 3D porous graphene coatings with pores
ranging between 1-10 µm between graphene flakes. Colloidal nanoparticles were used as template scaffolding which produced a hollow free-standing 3D graphene structure (500 nm). While each of these methods provide effective means for creating a highly porous 3D graphene architecture, they do not provide facile routes to pattern the solution-phase graphene into electrodes and in many cases are not stable (i.e., the architectures collapse in the air).

Simple, low-cost methods such as inkjet, aerosol, and screen printing provide simple fabrication methods for patterning solution-phase graphene into highly defined graphene electrodes; however, these graphene surfaces typically have little ECSA as graphene flakes are tightly compact exposing predominately their basal planes. Various techniques have been employed to alter the graphene flake orientation, for example, Choi et. al., incorporated nafion polymers into the graphene solution which made a hydrophobic (contact angle ~161°) rough surface, however, the graphene film lacked conductivity (>10kΩ/sq). Hersam and coworkers used polymer-phase inversion to tailor the porosity of graphene, similarly, the increase in porosity lead to a decrease in conductivity (~1000 S/m at 15% glycerol). Alternatively, we have demonstrated that secondary post-processing methods such as laser annealing can significantly increase the conductivity (~100 Ω/sq) while simultaneously enhancing the electroactive surface area of graphene by nano/micro structuring pores into the graphene by orientating superficial graphene flakes vertically. However, these methods do not make macrosized pores in the graphene surface or micropores in the graphene lattice structure while retaining electrical conductivity. Macropores in the graphene surface facilitate enhanced diffusion of substrates towards and away from the active sites (defect sites and edge planes in the graphene). Therefore, adding the additional three-dimensional hierarchy/architects of different pore sizes, as exhibited in these developed
electrodes for example, has been shown to result in higher electroactive surface area and enhanced catalytic activity.\textsuperscript{33-34}

Herein we demonstrate a facile manufacturing process of patterning solution-phase graphene electrodes with high electroactive surface area utilizing different orders of pore sizes \textit{via} salt impregnated inkjet maskless lithography (SIIML) and CO$_2$ laser annealing. This technique modifies the newly developed IML method for rapid prototyping highly defined graphene films by incorporating salt crystals as porogens to increase the ECSA.\textsuperscript{10,32} Sieved salt powders have been demonstrated in the past to act as efficient porogens for 3D printed resins,\textsuperscript{35-37} polymer-nanotube scaffolds for bone formation\textsuperscript{38} and porous carbon powders for gas absorbance.\textsuperscript{39} Similarly, we demonstrate that salt crystals can act as a hard template porogens for macrosize (< 25 to ~250 µm) defects in 2D thin film electrochemical devices. We also demonstrate for the first time, the use of a CO$_2$ laser to anneal patterned solution-phased graphene which etches microsize (~100 nm - 2 µm) pores in the graphene surface. This high ECSA graphene surface displayed enhanced electrochemical performance as demonstrated by an electrochemical pesticide biosensor with high sensitivity (12.4 nA/nM) and low limit of detection (0.6 nM) to paraoxon (a model organophosphate pesticide). Furthermore, we show that this multidimensional porous graphene surface is a platform technology that can be applied to electrochemical devices beyond biosensors, including energy storage, as demonstrated by an electrochemical supercapacitor fabricated \textit{via} the SIIML process which portrays high energy and power density (0.25 mWh/cm$^3$ at 0.3 W/cm$^3$).
Methods and Materials

Chemicals and Reagents

Completely chemically reduced single layer graphene oxide (referred to henceforth as graphene) was purchased from ACS Materials (GN1P0005). Sodium chloride (NaCl) was purchased from Sigma-Aldrich, ground using mortar and pestle, and filtered into different sizes with 8 in. wire cloth sieves. Acetylcholinesterase (ACHE) from *Electrophorus electricus* was purchased from Sigma Aldrich as lyophilized powder (200-1000 units/mg) and was used without any further purification. ACHE ink aliquots were made by dissolving the enzyme (2 mg) in 1 mL of 50% glycerol and 50% 1X phosphate-buffer solution (0.1 M PBS, pH 7.0), aliquoted into 25 µL, and stored in a -80°C freezer until use. All pesticides (including paraoxon) were obtained from Chem Service. All other chemicals and solvents were purchased from Sigma-Aldrich and were used without any other purification unless otherwise stated. Water was filtered through a B-Pure Water Purification system (resistivity, 18.2 MΩ/cm²).

Electrode Fabrication

Printing Graphene *via* Salt Impregnated Inkjet Maskless Lithography (SIIML)

Graphene electrodes were manufactured by modifying the inkjet maskless lithography (IML) method published earlier. A highly concentrated graphene ink was fabricated by probe sonicated 10 mg/mL ethylcellulose, 10 mg/mL nitrocellulose, and 20 mg/mL graphene in 200 mL of acetone for 4 hours. Ethylcellulose and nitrocellulose have been shown to help suspend the graphene flakes in solvents, improve the adhesion of the graphene to the surface, and carbonize upon annealing which reducing flake to flake resistance. Sieved microsized salt crystals (<25 to ~250 µm) were then incorporated into the graphene ink which made macrosized pores into the patterned graphene surface, henceforth referred to as salt impregnated inkjet maskless lithography (SIIML). In summary, a sacrificial polymer layer
(20% formaldehyde resin and 80% cyclohexanone) was inkjet printed using a Dimatix Material Printer (DMP-2850, 10 pL nozzles) onto heat treated polyethylene terephthalate (PET, [kemafoil, Coveme TSL W]). A highly concentrated graphene ink was spin-coated (1000 rpm for 30 sec) over the patterned sacrificial layer and post-baked for 10 min under a heat gun (Steinel #HB1750K) at a distance of 18 inches. An additional layer of graphene ink was then spin-coated over the sample that included discrete-sized salt crystals (ground using mortar/pestle and sieved into distinct sizes, 25-50 µm unless noted otherwise). The graphene ink was typically used immediately, but only slight settling of salt was observed after a week and was easily vortex mixed back to a suspension for re-use. The substrate was then again post-baked to remove solvents and increase graphene adhesion, followed by impinging the entire surface with an acetone wash bottle to remove the sacrificial layer. This process created a highly defined electrode pattern with salt crystals impregnated within the surface.

**CO₂ Laser Annealing**

The SIIML graphene electrodes were laser annealed using a CO₂ laser engraver (Orion Motor Tech 40 W). The raster engraving rate was set to 350 mm/s for all experiments, and the power of the engraver was set to 6.2% (estimated at 6.2 W) with a spot size ~150 µm, unless otherwise specified. These settings were found to properly anneal the graphene (carbonize surfactants and “weld” flakes together) which increased conductivity. Using a CO₂ laser to anneal and etch the surface of the graphene revealing the salt crystals, facilitating the “opening” of the macropore (Figure D.1). Using a CO₂ laser also etched the individual graphene flakes making microsized pores (100 nm – 2 µm) in graphene surface which further enhanced the catalytic abilities by providing additional edge defects (Figure D.2).
**Biosensor Design and Fabrication**

**ACHE Ink Preparation and Deposition**

SIIML electrodes were functionalized with ACHE using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDC/NHS) chemistry, which facilitated the functionalization of carboxyl groups to the amines within the ACHE enzyme. In summary, the CO₂ laser annealed SIIML electrodes were dipped in a solution of EDC/NHS mixture (50 mM EDC, 50 mM NHS) and incubated for 30 min on a tilter mixer. The electrodes were then rinsed with 1X PBS, excess water was blown off but not completely dried as the surface remained still “wetted”, this allowed for easier pipetting onto the slightly hydrophobic graphene surface. Next, 5 µL of the pre-aliquoted ACHE solution was immediately pipetted onto the surface and incubated overnight. Electrodes were washed 3 times in 1X PBS to remove any unbound ACHE before testing. Note for final inhibition pesticide sensor, a diluted concentration (97%) of ACHE was immobilized on the electrode surface which provides a higher inhibition signal output. This is because less enzyme coverage equates to lower concentrations of pesticides that are needed to inhibit said enzyme and hence change the biosensor signal output.

**Electro-analytical Measurements**

All electrochemical biosensor measurements and procedures were conducted on a CH instrument potentiostat (600E series) with a standard three electrode setup (single membrane Ag/AgCl reference electrode, platinum wire counter electrode, and CO₂ laser annealed SIIML graphene working electrode). Unless specified, each test was performed with 15 mL of 1X PBS buffer. For pesticide sensing, the biosensor was first tested in buffer to achieve baseline and ensure that solution did not contain contaminates that oxidize at 400 mV. The sensor was
then tested in 5 mM ACTH to acquire a base sensitivity to ACTH. The biosensor was then placed into the aqueous test solution with the pesticide for a minimum of 500 sec. During this incubation step, the pesticide diffuses to and permanently binds to ACHE, preventing it from catalyzing ACTH and therefore the product thiocholine from oxidizing on the electrode surface. The sensor is then rinsed with buffer and retested in the same 5 mM ACTH to acquire the decrease in amperometric response (referred to henceforth as inhibition sensitivity), see representative amperometric graph (Figure D.3).

**Supercapacitor Design and Fabrication**

**SIIML Graphene Supercapacitors Fabrication**

Graphene supercapacitors were designed similar to the SIIML graphene biosensor transduction layer, however, the rotation rate of the spin-coating process was increased from 1000 rpm to 2000 rpm so a thinner layer of graphene (~1.5 µm/layer) was deposited. A thinner layer (3µm, verified through ion milling through sample and measuring cross-sectional height using SEM) was used in order to increase the overall power and energy density by using a smaller supercapacitor volume. A dry gel electrolyte was used for the supercapacitor consisting of poly(vinyl alcohol) and phosphoric acid (PVA/H₃PO₄).³⁰ In Short, 6 mL of DI water was mixed with 3 mL isopropyl alcohol (IPA) and 1 mL concentrated H₃PO₄. The solution was then placed on a hotplate (80°C) and 1g PVA (poly(vinyl alcohol)) was slowly added until completely dissolved. A 25 µL aliquot of the solution was then pipetted onto each of the IDEs, which were placed in a dessicator for one hour to remove any air bubbles and insure that the electrolyte properly wetted the porous graphene electrode. Finally, the electrodes were dried in ambient air overnight.
Electrochemical Supercapacitor Characterization

The SIIML and IML (no salt) graphene supercapacitors were characterized by cyclic voltammetry (CV) and galvanostatic charge-discharge (potentiometry) experiments with two electrode (shorting counter and reference) setup. Capacitance at different current densities were calculated using the discharge curves obtained from galvanostatic graphs and Eq. 1.

\[
C = \frac{I \cdot \Delta t}{\Delta U}
\]

where I is the applied current (amps), \(\Delta t\) is the discharge time (sec), and \(\Delta U\) is the discharge voltage after self-discharge (IR drop of the electrode) is removed. Similarly, areal surface area capacitance (Eq. 2) and volumetric capacitance (Eq. 3) are calculated by dividing the capacitance by the areal surface area (\(A = 0.25\text{cm}^2\)) and the total volume of the IDE (\(V = 0.000075\text{cm}^3\)), this includes active electrode area and space between electrodes.

\[
C_A = \frac{C}{A} \quad \text{&} \quad C_V = \frac{C}{V}
\]  

The volumetric energy density (\(E_V\)) and power density (\(P_V\)) were calculated using Eq. 4 and 5.

\[
E_V = \frac{C_V \Delta U^2}{2 \cdot 3600 \cdot V} \quad \text{&} \quad P_V = \frac{C_V \Delta U^2}{2 \cdot V \cdot \Delta t}
\]  

Electrochemical Surface Area (ECSA) Calculations

All ECSA experiments were conducted in 5 mM ferri/ferrocyanide in 1X PBS. CVs were conducted with three different scan rates (25, 50, and 100 mV/s) and were used to calculate the ECSA using the Randle-Sevcik equation (Eq. 6). \(^{43-44}\) refer to representative graphs, Figure D.4.

\[
I_p = 2.69 \times 10^5 AD^{1/2}n^{3/2}v^{1/2}C
\]
Where $A$ is the effective electroactive surface area ($\text{cm}^2$), $D$ is the diffusion coefficient ($7.6 \times 10^{-6} \text{ cm}^2/\text{s}$), $n$ is the number of electrons in the Faradaic reaction ($n = 1$), $v$ is the scan rate ($\text{V/s}$), $C$ is the concentration of the bulk redox species (5 mM), and $I_p$ is the current at the oxidation peak. Note: the width between the anodic and cathodic peak often increased with higher scan rates which is often seen in very porous materials.\textsuperscript{10}

**Field Emission SEM Images**

Field emissions scanning electron microscopy (FESEM) images were acquired using a FEI Quanta 250 FESEM. All images were magnified to 500x, 1500x, 5000x, or 15,000x using backscattering electrons, spot size of 3.0, and with a 10 kV potential. A 2 nm conductive coating of iridium was evenly coated over all samples with a turbo-pump sputter coater to improve surface imaging by preventing surface charging and hinders electrons from penetrating into the carbon material leading to poor surface contrast.

![Field Emission SEM Images](image)

**Figure 6.1** Schematic diagram depicting the fabrication process steps of SIIML graphene electrodes. (a) A sacrificial polymer layer is inkjet printed as the negative of the final pattern. (b) Concentrated graphene ink impregnated with salt crystals is spin-coated. (c) The entire surface is impinged with acetone to remove the sacrificial layer, patterning the graphene. (d) A CO\textsubscript{2} laser anneals the graphene which increases conductivity, etches micropores, and reveals the salt crystals. (e) Electrodes are finally rinsed with water to remove the salt porogen, making macrosized pores within the graphene surface.
Results and Discussion

Overview of SIIML of Graphene Electrode Fabrication

Highly electroactive graphene transduction electrodes were fabricated by a modification to the newly developed IML process\textsuperscript{10,32} which utilizes solution-phase graphene instead of CVD grown graphene eliminating the need for high temperature annealing (>1000°C); the latter is costly and requires silicon wafer substrates which are insufficient for large scale roll-to-roll production.\textsuperscript{45} This manufacturing method, coined salt impregnated inkjet maskless lithography (SIIML), incorporates salt crystal as porogens in the graphene ink during IML manufacturing creating macrosized pores in the graphene. Additionally, CO$_2$ laser annealing is employed which creates microsized pores in the surface of the graphene.

First, a polymer sacrificial layer is inkjet printed onto a disposable, low-cost, and flexible substrate (PET), Figure 6.1a. A highly concentrated graphene ink is then spin-coated over the entire polymer sacrificial layer, followed by an additional graphene ink layer impregnated with salt crystals. The entire sample is then post-baked with a heat-gun to remove solvents and increase graphene adhesion to the substrates, Figure 6.1b. As the salt crystals are ionic solids, they do not ionize in the non-polar organic solvent and when patterned form a hard template (porogen) for macrosized pores. The entire graphene surface is then impinged by an acetone wash bottle which removes the underlying polymer sacrificial layer, patterning a highly-defined graphene electrode, Figure 6.1c. As acetone is not highly polar, the salt remains in its crystalline form as a hard template beneath the graphene surface. The graphene is then treated with a CO$_2$ laser which anneals the surface, removes remaining solvents, carbonizes surfactants, and “welds” graphene flakes together.\textsuperscript{9} The CO$_2$ laser also etches micropores into the graphene surface enhancing electrochemical activity by producing
additional edge planes in the graphene surface. Furthermore, laser annealing also etches the graphene surface revealing the salt crystals, opening the surface for pore formation, Figure 6.1d and Figure D.1. Finally, the electrode is rinsed with DI water which removes the salt porogen crystals, forming macrostructured pores in the graphene surface, Figure 6.1e. These macropores improve electrochemical sensing by providing increased analyte diffusion rates and increased ECSA.

**Overview of Pesticide Biosensor Mechanism and Fabrication**

An electrochemical pesticide biosensor was designed in order to evaluate the enhancement in electrochemical activity of SIIML graphene and to refine manufacturing properties (*i.e.* laser annealing power, salt size, and salt concentration). The SIIML graphene electrodes were biofunctionalized with the enzyme ACHE *via* EDC/NHS chemistry, Figure 6.2a. As previously mentioned and discussed in more details later, laser annealing graphene in ambient air conditions creates a high ECSA but also provides increased defect/holes in the graphene lattice structure which are rich in oxygen functional groups (epoxide sites, hydroxyl, carboxyl, etc.). The carboxylic groups provide unique immobilization sites for functionalization *via* EDC/NHS deprotonation mechanisms.46

This immobilization strategy (EDC/NHS) was chosen as it has been widely studied, covalently crosslinks with “zero order” length to carboxylic functional groups, is water soluble (no prior organic solvent dissolution necessary) and provides excellent subsequent stability to enzymes.47 First, EDC reacts with carboxyl groups (formed through laser annealing) to make an O-acylisourea intermediate ester. Second, to increase stability of this short-lived ester, NHS is added to form a stable amine-reactive NHS ester, which increases immobilization efficiency
of EDC coupling reaction. Finally, ACHE is then bound to the ester by a free amine in the enzyme, Figure 6.2a.

The use of ACHE for inhibited pesticide biosensing has been demonstrated many times in the literature, but for clarity, Figure 6.2b illustrates its basic mechanism. The biosensor operates by hydrolyzing the molecule ACTH which produces acetic acid and thiocholine. Two thiocholine molecules are oxidized at the graphene surface at an applied potential of 400 mV, Figure D.5; this reaction produces dithiocholine and two electrons. The thiocholine oxidation reaction is used as a baseline amperometric signal for sensing the inhibition of ACHE by pesticides. As increasing concentrations of pesticides are added to the solution, more ACHE is inhibited which prevents the formation of thiocholine, consequently this reduces the oxidation and amperometric current (see representative amperometric graphs in Figure D.3).

Figure 6.2 Schematic diagram of ACHE biosensor depicting the functionalization strategy for pesticide biosensing using EDC/NHS. (a) From Left to right: EDC reacts with carboxyl groups to make an O-acylisourea intermediate ester. NHS reacts to form a stable amine-reactive ester. Finally, NHS ester binds ACHE to graphene surface through available amines in the enzyme. (b) Basic operations of ACHE pesticide biosensor. From Top to bottom: ACTH reacts with ACHE and produces acetic acid and thiocholine. Thiocholine (2x) is oxidized by the graphene electrode and produces dithiocholine and two electrons. Paraoxon (model pesticide/organophosphate) inhibits ACHE and prevents the catalysis of ACTH. Note: size not to scale.
Laser Annealing Process

Herein, we demonstrate for the first-time laser annealing solution-phase printed graphene with a CO\textsubscript{2} laser. Similar to our previously published work with a Nd:YAG\textsuperscript{9, 49} and diode laser,\textsuperscript{10, 32} annealing the SIIML graphene with a CO\textsubscript{2} laser also significantly increased the conductivity by “welding” individual graphene flakes together and carbonizing the surfactants (ethyl/nitrocellulose) which reduces flake-to-flake resistance. The power of the CO\textsubscript{2} laser was incrementally increased from 5\% to 6.4\% (estimated as 5-6.4 W) which significantly lowered the sheet resistance of the graphene nearly 3 orders of magnitude (>10000 Ω/sq to ~50 Ω/sq) (Figure 6.3a (blue)). We further confirm the carbonizing of the surfactants by XPS which shows a decrease in the sp\textsuperscript{3} peak (green) which we attribute to ethyl/nitrocellulose, Figure 6.3b. Interestingly, unlike the diode laser or the Nd:YAG,\textsuperscript{9-10, 32} the CO\textsubscript{2} laser does not alter the graphene flake orientation; rather, it fuses graphene flakes together (5 W till 5.8 W) increasing the conductivity (~10 kΩ/sq to ~50Ω/sq, Figure 6.3a (blue)), then etches through the surface making microsize pores (Figure 6.3c). The surface roughness (electrochemical surface area/geometric surface area) of the laser annealed graphene begins to increase (Figure 6.3a (red)) as the resistance of the electrode decreases. The not annealed patterned graphene initially has a negligible surface roughness and high electrical resistance. When laser annealing at a power of 5.2 W the graphene surface roughness increased to 2.2. However, as the laser power increased past 5.2 W the graphene flakes begin to “weld” together making a flat plane which decreases the ECSA (surface roughness of 1.7 at 5.6 W) due to decreases in available edge planes. Further increasing the laser power not only “welds” the graphene flakes together promoting even higher conductivity but starts to etch through the basal planes of the graphene which leads to increased edge plane defects by making microsize pores (100 nm – 2 µm) in the graphene surface and consequently increasing the
electrochemical surface roughness (2.6 at a power of 6.2 W). The conductivity and surface area both decrease after 6.2 W as the graphene is etched through the surface and the substrate begins to degrade. Therefore, for all further experiments the CO$_2$ laser was set to 6.2 W with a 350 mm/s raster rate.

Raman spectroscopy was employed to analyze the atomic structure of the printed graphene and the effect of CO$_2$ laser annealing. Figure D.6 displays the Raman spectra collected from not annealed and increasing laser power annealed graphene (5.2-6.4W) with a backscattering geometry and 1064 nm Nd:YAG laser and a spot size of 1mm. Note that the spectra has been standardized so all samples have consistent G peak heights. The not annealed printed graphene displayed distinct D and G peaks which are characteristic for graphene and graphitic samples. However, the sharp 2D peak, typically associated with graphene is not visible. The loss of the 2D peak is probably due to the high concentration of surfactants within the printed graphene sample and possible impurities/functional groups on the graphene surface. However, as the laser power increases from 5.2W to 6.2W the 2D peak develops, indicating the formation of a graphene like structure by energetic photon irradiation. Furthermore, as the laser intensity increases, there is a decrease in (I$_D$/I$_G$) ratio (from 0.72 for not annealed graphene to 0.17 for 6.2 W) which suggests that the printed graphene forms a higher degree in edge-induced defects, which are conducive towards enhanced electrochemical reactivity.$^{9, 50}$ Additionally as the laser power increases there is an increase in (I$_{2D}$/I$_G$) ratio (from 0.23 for not annealed graphene to 0.78 for annealed graphene with a laser power of 6.2 W) which correlates with reported multilayer graphene structures.$^{51}$ At a laser power of 6.4 W, the ratios reverse as the samples are etching through the surface and there are possible formation of Van Der Waals bonds into more a graphitic nature. In summary, the emergence of a symmetric 2D
peak with an increase in \((I_G/I_D)\) and \((I_{2D}/I_G)\) ratio at higher laser powers demonstrates the carbon surface is more nanostructured or nanotextured with a higher amount of edge defects and the sp² honeycomb lattice of the surface becomes more favorable to classification as multilayered graphene.

It is interesting that we do not see a significant increase in graphene oxidation (Figure 6.3b) as we are laser annealing in ambient oxygen atmosphere as seen by our previous work with a diode laser. Instead there is approximately a consistent small oxide layer formed across all laser powers. These oxide functional groups are most likely superficial as oxidizing deep into the graphene electrode would decrease the electrode conductivity (not seen in Figure 6.3a), which may partially explain the lower sheet resistance of CO₂ laser annealing (~50 Ω/sq) compared to using a diode laser (~100 Ω/sq). In any case, the oxidation groups and defects in the graphene surface provides effective functionalization locations for both biomolecules or even nanoparticle nucleation locations, as we have shown previously.

![Figure 6.3 Effects of CO₂ laser annealing graphene. (a) Electrode sheet resistance (blue) and electrochemical surface roughness (red) versus increasing laser power. (b) XPS of not annealed, 5.8 W, and 6.4 W laser annealed graphene. (c) Representative SEM of laser annealed graphene surfaces with various powers showing the initial welding of graphene flakes at low power and then etching through the surface making microsized pores at higher powers.](image)
**Salt Impregnation Process**

The ECSA of the graphene electrodes was enhanced by incorporating macrosized pores via salt crystals in the graphene ink. Distinct sized salt crystals (ground using mortar and pestle and sieved into different sizes) were incorporated directly into the second layer of spin-coated graphene ink. Once dried, these salt crystals acted as porogens (hard templates) for macrosized pore formations in the graphene surface, and were subsequently removed by washing with DI water. Inks with various size salt crystals (100 – 250 µm, 75 – 100 µm, 50 – 75 µm, 25 – 50 µm, and < 25 µm, based on sieve mesh size) were made and cast with IML to better characterize the microstructuring and electrochemical enhancement of SIIML. The surfaces were imaged with SEM, Figure 6.4a, displaying the distinct pore structure which is controlled by the size of the salt crystals used. An electrode with no salt incorporated into the second spin-coated layer was used as a control sample and exhibit a relatively smooth surface with limited surface structures (apart from micropores formed from the laser annealing), Figure 6.4a and Figure D.7. As the salt crystal size decreases, a denser pore array is formed due to a higher salt suspension stability of the ink and the salt is able to form a tighter array when spin-coated on the surface.

This tighter pore array as well as the increased “surface-to-volume” effect (smaller pores will have higher surface area per vacancy ratio) results in an increase in surface area as the size of the pores decrease, Figure 6.4b (red). This effect is clearly seen by an over 50% increase in ECSA of electrodes made with 25 - 50 µm pores compared to electrodes made without salt pores. Interesting, after the pore size decrease below 25 µm, there is a significant decrease in ECSA. While it is not completely understood, we speculate that the size is approaching the microporous size made by laser annealing and is therefore mitigating the effect
of one or the other. Additionally, this decrease in ECSA could be from the decrease in perpendicular diffusion of substrate into the electrode which would decrease the electroactivity of the substrate.

Increasing the ECSA of the graphene by incorporating pores significantly enhanced the electroactivity of the electrode by providing additional sites for oxidation or reduction of the substrate. Moreover, the higher surface area of the porous graphene also provides additional binding locations for enzymes immobilization, which allow for improved substrate diffusion kinetics and consequently enhanced catalysis. Both of these effects improve the performance of the biosensor which increases its sensitivity to the analyte of interest. To test this, the electrodes were placed in a concentrated solution (5 mM) of ACTH with a working potential of 400 mV (versus Ag/AgCl). When ACTH reacts with ACHE, thiocholine is produced which oxidizes at the electrode. The corresponding increase in current due to the sensitivity to ACTH was graphed, Figure 6.4b (green). As the size of the pores decrease and the ECSA increases, there is a general increase in sensitivity to ACTH as expected. Variations from this trend (150% for 75-100 µm and 133% for 25-50 µm), are likely due to manufacturing errors (enzyme loading differences), experimental setup (sensors did not full equalize to baseline), or random/systematic errors. These results are further demonstrated by SIIML graphene biosensor with 25-50 µm being over 2 times more sensitive to the ACHE than without salt pores, Figure 6.4b (blue). For all further experiments, 25-50 µm salt particles were used as they exhibited the highest ECSA and was the most sensitive to paraoxon.
The concentration of the 25-50 µm salt/graphene ink suspension was varied to improve the pore array density of the graphene surface, Figure 6.5a. As the salt concentration increased, the pores in the graphene surface on average come closer together forming a denser pore array. At 50 mg/ml, the pores are fairly far apart, and a sparse array is formed (roughly estimated by visibly counting pores in SEM, ~75-125 pores/cm). When the concentration was increased to 250 mg/ml a network of pores are formed (~300-500 pores/cm), and at 500 mg/ml a very dense array of pores is present (~500-750 pores/cm). Note that as the concentration of salt increases and the salt packs closer together, the pore structures are harder to distinguish due to overlap. Additionally, as the salt concentration increases, the viscosity of the graphene ink also increases. At 500 mg/mL, the ink is much more viscous (making it more difficult to spin-coat) and so a thicker layer of graphene may have been applied which results in the salt not all lying flat against the bottom surface causing salt porogen overlap, Figure D.7.
As the pore structure in the graphene becomes denser, the biosensor electrode (functionalized with ACHE), becomes more sensitive to ACTH (Figure 6.5b), however, it appears that after 175 mg/ml, the anodic current relatively plateaus. Interesting, this trend is not completely observed with the inhibition sensitivity of paraoxon and after 175 mg/ml the sensitivity begins to decrease. This observation is not easily explained, but we speculate that the higher surface area increased the loading of the enzyme on the graphene, resulting in more enzyme that needed to be inhibited. As the concentration of 175 mg/ml yielded a porous graphene biosensor with the most sensitivity to ACTH and the signal response was most inhibited by paraoxon, all further experiments were conducted with this salt concentration.

Biosensor Performance Characterization

The electrochemical activity of SIIML graphene electrodes was demonstrated by manufacturing a pesticide biosensor using ACHE and refined parameters shown previously (6.2 W CO2 laser annealing, 50-75 µm salt pores, and 175 mg/mL salt concentration). First,
an array (4x10) of 3 mm diameter disk electrodes were manufactured using the procedure outlined in the **Overview SIIML Graphene Electrode Fabrication** (Figure 6.6a). A lacquer was then pipetted onto the stem of the electrode to isolate the working area (3 mm diameter disk) and biofunctionalization was carried out as described in the **Overview Pesticide Biosensor Fabrication**. The ACHE pesticide sensor was placed in a saturated solution (5 mM) of ACTH and amperometric current was recorded with changing potentials (Figure 6.6b). When the potential reached 200 mV (versus Ag/AgCl) there was an increase in anodic current due to the oxidation of thiocholine. This oxidation current increases as the applied potential approaches 400 mV then plateaus, therefore, an applied potential of 400 mV was chosen for the ACHE biosensor. This observation is in good agreement with CV scans that show an oxidation peak around 400 mV (Figure D.5) and similarly reported in the literature.\textsuperscript{52-53}

ACHE pesticide biosensors rely on the inhibition of the enzyme (paraoxon irreversibly binds to ACHE which prevents ACTH from catalyzing and hence thiocholine from oxidizing). Therefore, to test the amount of time required for organophosphates to diffuse to the electrode surface and bind to ACHE, we incubated the sensors in 1 µM of paraoxon and recorded the decrease in amperometric oxidation current (which correlates directly with the percent of ACHE inhibited), Figure 6.6c. After 100 seconds, there is roughly 50% inhibition of the sensor with large standard deviations; however, after 500 seconds, there is about 90% inhibition with low standard deviation and minimal change over the next couple of hundred seconds. Therefore, an incubation time of 500 seconds was used as a minimal time for sensor operation.

Graphene electrodes were made with (SIIML) and without salt pores (IML) for comparison. Increasing concentrations of ACTH (100 µM additions) were spiked into a buffer solution and the amperometric anodic current was recorded for both SIIML and IML. The
electrodes containing salt pores had a sensitivity to ACTH of 28.3 µA/nM while the electrodes without salt pores had a sensitivity of ~13.3 µA/nM, Figure 6.6d.

Finally, the SIIML were tested for pesticide sensing with paraoxon (a model organophosphate pesticide). It should be noted here that a diluted concentration of ACHE was applied to these pesticide biosensors (~2 U/sensor) as having a smaller concentration of enzyme results in higher sensitivity due to less enzyme that needs to be inhibited. An amperometric baseline of eight different SIIML ACHE pesticide biosensors were recorded in 5 mM ACTH which had an anodic current of 13.84 ± 0.3 µA. Each of the sensors were then placed in different concentrations of paraoxon (10 nM – 50 µM) and incubated for a minimum of 5 min, and then placed back in the same 5 mM ACTH solution (Figure 6.6e). The concentration versus inhibition current (decrease in anodic current) was graphed (Figure 6.6f) and showed a linear region up to 500 nM and then a plateau region shortly thereafter. The sensitivity of the linear region was calculated to be 12.4 nA/nM with a limit of detection of 0.6 nM (LOD = 3 Noise/Signal, noise defined as standard deviation of three electrodes without ACHE). Therefore, our sensor has a limit of detection below the tolerable drinking water equivalence level of the United States and Canada EPA (24 nM and 170 nM, respectively). There are many reported ACHE biosensors with lower limit of detection, even as low as 0.4 pM; however, these biosensors often employ complex methods to enhance their sensitivity and reduce noise such as metal nanoparticles, multilayer carbon nanotube-modified glassy carbon electrodes, co-enzymes, flow cells or microelectrodes.
Figure 6.6 ACHE pesticide biosensor characterization. (a) Photograph of patterned array (4x10) SIIML graphene electrodes on flexible PET substrate. (b) Effect of applied potential to amperometric response of 1 mM ACTH. (c) Plot of inhibition (%) according to incubation time. (d) Sensitivity to ACTH for SIIML (blue) and IML (red), inset: current versus concentration. (e) Amperograms of pesticide ACHE biosensor after incubating with various concentrations of paraoxon. Buffer baseline (black), 10 nM (red), 50 nM (green), 100 nM (yellow), 500 nM (blue), 1 µM (pink), 5 µM (aqua blue), 10 µM (grey), 50 µM (brown). (f) Corresponding current versus concentration, inset: magnification showing linear sensing range.

Supercapacitor Performance Characterization

To further demonstrate the enhanced electrochemical activity of SIIML electrodes, supercapacitors were designed. Supercapacitors offer promising energy storage opportunities for on-chip devices as they can be fabricated directly in line with the electronics. Supercapacitors store energy at the interface of the electrode and electrolyte; therefore, increasing the ECSA and conductivity of the electrode improves the efficiency and capacitance of the supercapacitor. Interdigitated electrodes (IDEs) were designed and patterned using the procedures outlined in the Overview SIIML Graphene Electrode Fabrication, and an array (2x4) of IDEs were patterned, Figure 6.7a. The SIIML graphene IDEs were then coated with PVA/H₃PO₄ gel which acts as a dry gel electrolyte. The performance of the SIIML graphene
supercapacitor was first examined with CVs of various scan rates (50 mV/s – black to 1000 mV/s – orange), Figure 6.7b. The CVs show a rectangular shape at low scan rates and only begin to distort in shape at very high scan rates (1000 mV/s) due to internal resistance. The capacitive current (due to double layer charging) of the SIIML graphene electrodes was extracted from the CVs and compared with IML graphene with no salt. The graphene with salt pores displayed enhanced double layer capacitance (37%) current as portrayed in Figure 6.7c. Galvanostatic charge-discharge profiles of the SIIML were then graphed which shows relatively symmetric triangular shape, but slowly loses symmetry at very low charging densities (0.01 mA/cm²). Based on the discharge rates, the capacitance was calculated and graphed versus the current density, Figure D.8. On average, the SIIML graphene had ~30% more capacitance than the devices without salt (IML).

Multiple supercapacitors can be connected in series (to boost voltage capabilities) or in parallel (to increase energy storage density) as illustrated and graphed in Figure 6.7e. When three electrodes are connected in series (blue) the voltage window increased by a factor of three with roughly the same charge-discharge times. In contrast, when three electrodes are connected in parallel, the charge-discharge times increase by a factor of three with the voltage window remaining constant. Therefore, these supercapacitors could easily be patterned in series or parallel to adapt for a specific application. To demonstrate that SIIML graphene patterning process for supercapacitor use, an array of supercapacitors was fabricated (3 in series and 5 in parallel) and were used to power a red LED (drop voltage ~1.8V). After charging the supercapacitor to 3 V, the LED was tested across the supercapacitor array and illuminated multiple times before the supercapacitor’s voltage decreased below 1.8V.
Finally, the SIIML graphene and IML graphene (no salt) energy density and power density were extracted from the galvanostatic discharge and graphed on a Ragone plot (Figure 6.7f). The graphene electrodes that contained salt pores demonstrated higher energy density due to the increase in ECSA which stored more charge at the graphene/electrolyte interface. Both the SIIML and the IML graphene interdigitated electrodes compared favorably with other carbon-based supercapacitors IDEs reported in the literature, with the SIIML outperforming the IML electrode (energy density of 0.25 mWh/cm³ and 0.17 Wh/cm³ at a power density of 0.3 W/cm³, respectively). Note that supercapacitors are strongly dependent on the electrolyte used, and by using a more efficient electrolyte, a higher energy and power density could be obtained.

Figure 6.7 Electrochemical performance of a SIIML supercapacitor. (a) Photograph of patterned SIIML graphene electrode with schematic of supercapacitor design. (b) Cyclical voltammograms with increasing scan rates (50 mV/s black to 1000 mV/s orange). (c) Scan rate versus current demonstrating linear relationship and enhanced capacitance of SIIML (red) over IML (blue). (d) Galvanostatic charge-discharge curves with different current densities (0.01 mA/cm²-orange to 0.2 mA/cm²-red), inset: magnification showing near triangular shapes at higher current densities. (e) Galvanostatic charge-discharge graph of single supercapacitor (black), three electrodes connected in series (blue), and three electrodes connected in parallel (red). (f) Ragone plot of SIIML (red) and IML (blue) supercapacitor electrodes and various other carbon-based IDE supercapacitors; light green: inkjet printed single walled carbon nanotubes (SW-CNT), brown: boron doped laser induced graphene (B-LIG), pink: electrostatic spray deposited reduced graphene oxide/Carbon Nanotube (rGO/CNT), yellow: electrochemically reduced graphene oxide (ERGO), and dark green: methane plasma treated graphene oxide with gold collector (MPGO).
Conclusions

In conclusion, a micromanufacturing technique for enhancing the electrocatalytic activity of patterned solution-phase graphene was demonstrated with the use of a modified IML technique. This technique creates a multiscale porous architecture in the graphene surface. Macrosized pores (25 - 50 µm) are formed by utilizing salt crystals as porogens (hard templates) in the graphene ink while patterning, referred to as salt impregnated inkjet maskless lithography (SIIML). Additionally, microsized pores (100 nm - 2 µm) are etched into the graphene surface through CO$_2$ laser annealing, even on flexible heat sensitive substrates (PET). We demonstrate that SIIML is an effective tool for enhancing the electrochemical activity of graphene by fabrication of an electrochemical pesticide biosensor, which utilizes ACHE. Graphene electrodes with macrosized pores through salt impregnation outperformed their non-macropore counterparts (sensitivity to ACTH of 28.3 µA/nM to 13.3 µA/nM). The final SIIML graphene ACHE sensor had a wide linear sensing range (10 nM to 500 nM), low limit of detection (0.6 nM), and high sensitivity (12.4 nA/nM) to paraoxon which is below the tolerable drinking limit reported by the EPA.$^{54}$ Furthermore, we demonstrate that this method can fabricated complex geometries such as interdigitated electrodes for use in other non-biosensing related applications (i.e., an electrochemical supercapacitor). Critically, the SIIML graphene outperformed non-macroporous graphene by demonstrating ~30% higher capacitance. The SIIML supercapacitor demonstrated excellent energy density of 0.25 mWh/cm$^3$ at a power density of 0.3 W/cm$^3$, which was comparable to other carbon-based supercapacitors reported in literature. Both the electrochemical biosensor and supercapacitor demonstrate that increasing the electrochemical surface area of solution-phase printed graphene electrodes via SIIML and CO$_2$ laser annealing improves the performance of these electrochemical devices. Moreover, these manufacture techniques (SIIML and CO$_2$ laser annealing) are amenable to
thermally sensitive and flexible substrate materials; therefore, this method could be adapted to wearable technology for energy storage or textile-based biosensors as well as for potential creation of flexible supercapacitors on paper-based substrates. Finally, this high ECSA graphene transduction electrode can act as a platform technology for additional electrochemical sensors or supercapacitors, as well other electrochemical devices such as batteries, biofuel cells, dye sensitive solar cells, or electrode stimuli.

References


CHAPTER 7. GENERAL CONCLUSIONS

Present Work

The research results and conclusions presented in this dissertation serve to guide future research to improve the growing field of electrochemical biosensors, specifically with an application toward pesticide monitoring and detection. Moreover, it hopes to improve on the present body of knowledge in the following areas: 1) expand upon the usefulness of graphene in electrochemical biosensors, its printing techniques, and its current applications, 2) provide further insight into improving the catalytic activity of enzymes by strategically functionalizing to nanoparticles, 3) describes a new rapid prototyping solution-phase patterning technique (inkjet maskless lithography) which is able to print high resolution graphene, 4) details the design and operation of a low-cost pesticide sensor for sensitive detection of pesticides, 5) provides insight into enhancing the electroactivity and surface area of patterned solution-phase graphene by modifying the surface (functional groups, nanoparticle deposition, micro/nanostructuring via laser annealing, increasing graphene porosity via salt impregnation). Through this work, we demonstrate these attributes with the goal of designing a simple, low-cost electrochemical biosensor for in-field detection of pesticides. The enzymatic biosensors (OPH and ACHE) demonstrated excellent sensitivity (370 nA/µM and 28.3 µA/nM) and ultralow detection limits (3 nM and 0.6 nM). The sensors displayed excellent stability, longevity, and were selective in soil and water samples.

While this work demonstrated the rapid prototyping of a graphene electrochemical biosensor for the purpose of pesticide sensing, the manufacturing techniques and biosensor design can be incorporated into a variety of different fields. Therefore, the graphene electrochemical biosensor described in this work is a platform technology and could be
amendable to a verity of different biosensors applications and other electrochemical devices (e.g. healthcare screening, drinking water monitoring, bioterror agent detection, supercapacitor, batteries, biofuel cells, or electrode stimuli). \(^1\textsuperscript{-5}\) For example, a high energy density and high power density supercapacitor was designed and described in chapter 6 which showed 30\% higher capacitance with the inclusion of macropores via salt impregnation. The supercapacitor effectively illuminated an LED and as it was fabricated on flexible substrate could be integrated into a wearable device.

**Challenges and Recommendations For Future Work**

While this dissertation has demonstrated significant advances in the field of electrochemical biosensors, there are significant challenges that lie ahead. Graphene is a new allotrope of carbon (discovered in 2004) and substantial research is needed to further understand its physical, electrical, and electrochemical characteristics and how they apply towards electrochemical biosensors. Specifically, more research needs to be conducted to understand and devise new printing techniques to pattern solution-phase graphene. We have demonstrated that IML can pattern graphene with high resolution for rapid prototyping, but this method can be further improved to decrease feature size. Additionally, IML can be scaled up to mass production by integrating into roll-to-roll fabrication for industrial manufacturing applications, which would significantly lower the cost per device. Developing graphene inks with low surfactant or polymer binder concentration that are able to still print effectively could also improve conductivity and electroactivity. Incorporating conductive polymers as surfactants instead of insulative binders could also improve conductivity and possibly electroactivity.
While this dissertation and our past work has described and shown the effectiveness of using laser annealing to increase the conductivity of patterned graphene and increasing its electrochemical surface area, more research into the molecular dynamics and the physics behind the change in graphene morphology could dramatically increase sensitivity for biosensors. Specifically, research into using different wavelength lasers at different powers could give significant insight into the physical interaction of graphene flakes (e.g. tuning vertically, etching through the surface, “welding” together). For example, in this dissertation we have shown that using a diode laser (405 nm) anneals graphene flakes very similarly to our earlier work with a ND:YAG (355 nm) by tuning graphene flakes vertically. Contrarily, CO\textsubscript{2} laser annealing (10.6 \textmu m) etching through the graphene lattice structure making nanosized size (~100 nm) holes in the graphene without altering the graphene flake structure.

We have demonstrated through this dissertation that sensitive organophosphate pesticide biosensors can be made with ACHE and OPH, yet there are many other different types of pesticide (herbicides, fungicides, rodenticides, bactericides, other insecticides) that are often applied to farmer’s fields. Developing a multiplex pesticide biosensor that incorporated various other types of biological recognition agent would make it possible to selectively determine which type of pesticide is active and at what quantity. For example, Solna et. al. has demonstrated that through an 8 working electrode multiplex sensor, organophosphates, carbamates and phenols could be selectively identified in wastewater samples. Multiplex biosensors are still in their infancy and further research into incorporating multiple different biorecognition agents could increase their usefulness in identifying various analytes. Similarly, while we have shown that our designed electrochemical biosensors work in real world samples (tap water, river water, soil slurry), further research into field testing the
devices is needed. Our graphene biosensors demonstrated very accurate results (within 10 percent) when tested with these real-world samples in the lab that were modified to the correct pH, ionic concentration and temperature, but testing these sensors directly in the field is required.

Finally, developing a disposable biosensor that can integrate directly onto a reusable microfluidic cartridge could have significant impact on a variety of sensing applications. For example, a multiplex tape biosensor that can selectively monitor different types of pesticides in soil samples would provide farmers with a useful system to monitor chemical application level in-field. Incorporating a reusable microfluidic would allow for easier filtering of soil particles while simultaneously increasing the biosensors sensitivity by decreasing the distance of the substrate diffusion layer. A biosensor that is patterned on adhesive tape could be used as a disposable, low-cost flexible tape sensor that can easily adhere and seal itself to the microfluidic. After sensing, the farmer could then easily peel off the tape sensor and apply a new one.

References

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APPENDIX A. ENHANCED ACTIVITY FROM PHOSPHO-TRIESTERASE TRIMER GOLD NANOPARTICLE BIOCONJUGATES FOR PESTICIDE DETECTION – SUPPLEMENTAL FIGURES

Figure A.1 Absorbance spectrum acquired at 405 nm for varying ratios of PTE$_3$-AuNP (20 nm AuNP fixed 0.5pM AuNP concentration). Comparison between varying ratios of PTE$_3$-AuNP (solid) and unbound PTE$_3$ (dashed) at equivalent concentration as listed in the legend. Paraoxon (substrate) concentration fixed at 5000µM. Absorbance data is initially scaled/translated to zero (at time zero) to highlight linear slope.

Figure A.2 Absorbance spectrum acquired at 405 nm for 1:1 PTE$_3$:AuNP (0.5 pM:0.5 pM) for 5, 10, and 20 nm AuNP at various concentration of paraoxon as delineated by the legend. Absorbance data is initially scaled/translated to zero (at time zero) to highlight linear slope.
Figure A.3 Absorbance spectrum acquired at 405 nm for fixed concentration PTE$_3$ enzyme (1.1pM) with varying concentration of AuNP (dia. 20 nm). Comparison between varying ratios of PTE$_3$-AuNP and equivalent concentration of free PTE$_3$ (dotted) as listed in the legend. Paraoxon (substrate) concentration fixed at 5000µM. Absorbance data is initially scaled/translated to zero (at time zero) to highlight linear slope.

Figure A.4 Absorbance spectrum acquired at 405 nm for 1:1 PTE$_3$:AuNP (dia. 20nm, 1.1 pM:1.1 pM, solid lines) and free PTE$_3$ (1.1pM, dotted) tested at various concentration of paraoxon as delineated by the legend. Absorbance data is initially scaled/translated to zero (at time zero) to highlight linear slope.
Figure A.5 Absorbance spectrum acquired at 405 nm for 1:1 PTE₃:AuNP (1.1 pM:1.1 pM) for 5 nm (dotted), 10 nm (dashed), and 20 nm (solid) AuNP at various concentration of paraoxon as delineated by the legend. Absorbance data is initially scaled/translated to zero (at time zero) to highlight linear slope.
Figure B.1 Optical images of resultant inkjet printed sacrificial layer. (a) 50 µm circles of sacrificial layer with 50 µm spacing between drops. (b) Printed sacrificial layer in IDE design with 50 µm width and 25 µm spacing. (c) Sacrificial layer in IML logo pattern. (d) Magnification of patterned IML Logo. IML Logo reprinted with permission from John Hondred. Copyright 2017.

Figure B.2 Annealing of patterned graphene after IML. Blue: Thermal annealing of graphene with increasing temperatures on Si/SiO₂ wafer. Red: Laser annealing of graphene with increasing laser time on heat treated PET.
Figure B.3 XPS graphs of IML graphene on heat treated PET. (a) without annealing and (b) after laser annealing in an ambient oxygen atmosphere.

Figure B.4 Confocal microscope images of graphene line with average cross-sectional height on Si/SiO$_2$ wafer.
Figure B.5 (a) Graphene and (b) silver IML patterns created using inkjet maskless lithography (IML) technique on heat treated PET. IML Logo reprinted with permission from John Hondred. Copyright 2017.

Figure B.6 Interdigitated electrode (IDE) design of graphene electrodes.
Figure B.7 SEM of graphene IDE created with IML on a Si/SiO$_2$ wafer. (a) Cross-section of graphene, scale bar 1 µm. (b) Horizontal angled view of graphene IDE, scale bar 200 µm. (c) Vertical angled view of IDE, scale bar 200 µm. (d) Magnified vertical angled view of graphene, scale bar 5 µm.

Figure B.8 Graphene IML logo on various substrates: (a) kemafoil (heat treated PET), (b) silicon wafer, (c) clear PET, and (d) Polyimide tape (Kapton®). IML Logo reprinted with permission from John Hondred. Copyright 2017.
Figure B.9 Graphene lines on heat treated PET after IML patterning. (a) Graphene flakes (red arrows) fusing together suspending over the sacrificial layer and not completely removed in acetone bath. (b) Patterned graphene after sonication or impingement from acetone wash bottle with no unwanted graphene flakes.

Figure B.10 Three electrode setup for hydrogen peroxide sensing using 3D printed vial to standardize testing.
APPENDIX C. PRINTED GRAPHENE ELECTROCHEMICAL BIOSENSORS FABRICATED BY INKJET MASKLESS LITHOGRAPHY FOR RAPID AND SENSITIVE DETECTION OF ORGANOPHOSPHATES - SUPPLEMENTAL FIGURES

Figure C.1 SEM of laser annealed graphene surface, with various carving (raster) speeds with various magnifications.

Figure C.2 SEM of electrodeposited platinum nanoparticles on laser annealed graphene, conducted with various number of platinum cycles with various magnifications.
Figure C.3 Effects of pH on paraoxon biosensor. Data extracted from amperometric experiments. a) Sensitivity to paraoxon vs. pH. b) Corresponding detection limit (3σ) vs. pH.

Figure C.4 Effects of applied potential to working electrode (vs. Ag/AgCl). Data extracted from amperometric experiments. a) Sensitivity to paraoxon vs. applied potential. b) Corresponding detection limit (3σ) vs. applied potential.
Figure C.5 Sheet resistance of laser annealed IML-PGE based on carving time.

Figure C.6 Cyclic voltammetry of various working electrode materials in 5 mM ferro/ferricyanide with various scan rates (5-50 mV/s). Inset: Randles-Sevcik plot: anodic and cathodic currents vs. with square root of scan rate. (a) SPCE, (b) IML-PGE [Top: 20 ms carving rate, Middle: Not annealed (0 ms), Bottom: Over annealed (100 ms)], (c) PtNPs-IML-PGE.
Figure C.7 Cyclic Voltammograms of various working electrode materials in buffer (gray) and 1mM p-nitrophenol (green, blue, red). a) Carbon, b) Laser annealed graphene, c) Platinized, laser annealed graphene hybrid.

Figure C.8 Effects of glutaraldehyde concentration in PTE ink. Data extracted from amperometric experiments. a) Concentration vs. current with increasing glutaraldehyde concentration. b) Histogram depicting sensitivity to paraoxon based on varying percentage of PTE.
APPENDIX D. ENHANCED ELECTROCHEMICAL BIOSENSOR AND SUPERCAPACITOR WITH 3D POROUS ARCHITECTURED GRAPHENE VIA SALT IMPREGNATED INKJET MASKLESS LITHOGRAPHY - SUPPLEMENTAL FIGURES

Figure D.1 SEM of Salt impregnated pores into spin coated graphene ink: **Left**: Before laser annealing showing salt in completely coated in graphene flakes. **Center**: After thermal annealing and rinsing showing salt has been removed but pore is not opened. **Left**: After laser annealing showing etching of surface opening the pore into the surface of the graphene.

Figure D.2 SEM of graphene surface with increasing laser annealing powers (5 W to 6.4 W, raster rate fixed at 350 mm/s) and various magnifications (1500x, 5000x, and 15000x). From not annealed to 5.8 W, graphene flakes are smoothed and welded together and from 5.8 W to 6.4 W, CO$_2$ laser begins to etch through the surface making microsized pores.
Figure D.3 Representative amperometric graph depicting basic ACHE biosensor data extraction.

Figure D.4 Representative cyclic voltammetry of SIIML graphene electrodes in 5 mM ferro/ferricyanide with various scan rates (5-50 mV/s). **Inset:** Randles-Sevcik plot: anodic and cathodic currents vs. with square root of scan rate.
Figure D.5 Cyclical voltammetry showing increase in ACTH concentration

Figure D.6 Raman spectra of not annealed graphene and increasing CO$_2$ laser powers.
Figure D.7 Confocal microscopy photos of not annealed graphene, laser annealed graphene, and macroporous SIIML graphene along with corresponding surface profiles.

Figure D.8 Calculated capacitance of supercapacitors from galvanostatic discharge of SIIML and IML (without salt) of supercapacitor devices.