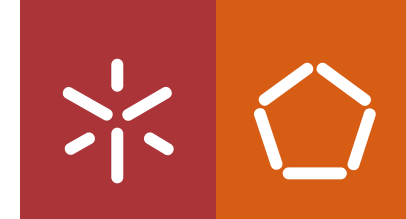


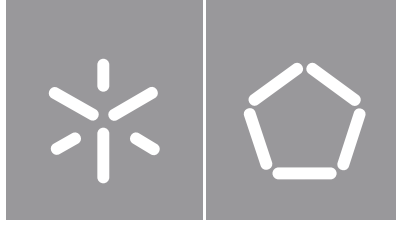


Mafalda Inês Gonçalves de Abrantes

**Graphene field-effect transistors
functionalization for
neurotransmitter biosensing**

Universidade do Minho
Escola de Engenharia





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Dissertação de Mestrado

Mestrado Integrado em Engenharia Física

Dispositivos, microssistemas e nanotecnologias

Trabalho efetuado sob a orientação do

Professor Doutor Luís Ricardo Monteiro Jacinto

e do

Professor Doutor João Pedro dos Santos Hall

Agorreta de Alpuim

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ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisors, Prof. Dr. Luís Jacinto and Prof. Dr. Pedro Alpuim, for the support, constant availability, and guidance throughout the thesis.

Most of this thesis work was carried out at the International Iberian Laboratory, and so I must thank the 2DMD (2D Materials and Devices) group members for all valuable input.

I also would like to thank the ICVS' researchers (Life and Health Sciences Research Institute) that assisted me with experimental procedures on the later part of this thesis.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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RESUMO

A compreensão de como o cérebro funciona é fundamental para o desenvolvimento de novos métodos de diagnóstico e terapêutica para as doenças que afetam o cérebro. O grafeno, devido às suas propriedades únicas, tem sido exponencialmente utilizado para novas aplicações biomédicas e é um material emergente para biossensores, incluindo no cérebro.

A dopamina é um neurotransmissor, a mensagem química produzida pelas células no cérebro, envolvida em várias funções críticas como o controlo motor e a aprendizagem. Logo, alteração dos níveis de dopamina está relacionada com várias doenças que afetam o cérebro, incluindo Doença de Parkinson, esquizofrenia e abuso de substâncias. De forma a obter um método de deteção para dopamina mais seletivo e sensível, neste trabalho foi desenvolvido um novo sensor bio eletrónico.

O canal de grafeno mono-camada de transístores de efeito de campo de porta líquida foi funcionalizado com uma sonda, o aptâmero de DNA específico para a dopamina. Quando o aptâmero se liga à dopamina, a redistribuição da carga eletrónica na superfície do grafeno gera uma mudança no campo elétrico que altera a condutividade eletrónica no canal e a resposta geral do dispositivo. Seguindo as alterações da condutividade com a adição de concentrações conhecidas de dopamina, definição das curvas de calibração dos sensores foi possível. Para tal, experiências de deteção de dopamina foram realizadas *in vitro* em solução salina de fosfato tampão e em líquido cefalorraquidiano artificial. No primeiro foi obtido um limite de deteção de 1 aM, o mais baixo reportado para um sensor de dopamina e no segundo o limite foi de 10 aM. A seletividade dos sensores também foi testada comparando as respostas da dopamina aos seus precursores, como L-Dopa e L-Tirosina, e a agentes interferentes como ácido ascórbico. O uso de um aptâmero específico para dopamina permitiu uma excelente seletividade, pois a resposta dos sensores a outras moléculas foi negligenciável. A excelente seletividade e sensibilidade dos nossos sensores permitiram também a realização de experiências *ex vivo* com amostras extraídas de murganhos. Os nossos sensores foram utilizados para demonstrar que a reserpina, uma droga que exauria a dopamina das sinapses neuronais, foi eficiente na redução do conteúdo de dopamina no líquido cefalorraquidiano de murganhos injetados com reserpina em 5 vezes quando comparado controlos. Foi também confirmada a habilidade dos nossos sensores em detetarem dopamina em meios complexos, através da adição de concentrações de dopamina artificial a homogeneizado de cérebro de murganhos tratados, tendo sido obtido um limite de deteção de 0.1 fM.

Os resultados inovadores permitem-nos considerar experiências futuras num ambiente *in vivo*.

Palavras chave: Aptasensor, dopamina, grafeno, transístor, baixo LOD

ABSTRACT

The understanding of how the brain works is fundamental for the development of new diagnostic and therapeutic methodologies for brain disorders. Graphene, due to its unique properties, is being increasingly used in a wide range of novel biomedical applications and is an emerging material for biosensing, including in the brain.

Dopamine is a critical neurotransmitter, the chemical messages produced by brain cells, is dopamine, which is involved in various functions from motor control to learning. Thus, dopamine dysfunction underlies several brain disorders, including Parkinson's Disease, schizophrenia and substance abuse. Striving to obtain a more sensitive and selective method of detection for dopamine, we engineered a new bioelectronic sensor for dopamine detection in this work.

Electrolyte-gated graphene field-effect transistors' monolayer graphene channel was functionalized with a molecular linker and a probe, a dopamine-specific DNA aptamer. When this receptor binds with dopamine, the redistribution of electronic charges on the graphene surface generates a change in the electric field which alters the electronic conductivity in the channel and overall device response. By tracking the changes in conductivity with the addition of known concentrations of dopamine calibration curves for our sensors were established. To do so, dopamine detection experiments were carried out *in vitro* in phosphate buffered saline solution and artificial cerebrospinal fluid electrolytic solutions. In PBS the obtained limit of detection (LOD) was of 1 aM, the lowest ever reported for a dopamine sensor, while in aCSF the LOD was of 10 aM. The sensors selectivity was also tested by comparing responses to dopamine with responses to dopamine precursors such as L-DOPA and L-tyrosine, and biological interferents as ascorbic acid. The use of a dopamine specific-aptamer allowed an outstanding selectivity, with the sensors producing negligible responses to the other tested molecules. The excellent selectivity and sensitivity of our sensors allowed us the performance of *ex vivo* experiments with samples extracted from mice. Our novel sensors were used to show that reserpine, a drug that can deplete dopamine from brain synapses, was effective in reducing dopamine content in cerebrospinal fluid (CSF) extracted from reserpine injected mice by 5-fold compared with controls. Taking advantage of brain homogenate samples that were depleted of dopamine by the action of reserpine, the ability of our sensors to perform detection in such a complex media was confirmed by adding known concentrations of dopamine to these samples. Although an expected loss of sensitivity was observed, the LOD in these complex samples was of 0.1 fM.

These ground-breaking results allow us to consider further experiments in an *in vivo* setting.

Keywords: Aptasensor, dopamine, graphene, transistor, lowest LOD

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LIST OF ABBREVIATIONS, INITIALS AND ACRONYMS

aCSF – Artificial cerebrospinal fluid	FET – Field-effect transistor
ADC – Analog-to-digital converter	FLA – Lateral flow assay devices
Al - Aluminum	FRET – Fluorescence resonance energy transfer
Ar – Argon	FSCV – Fast-scan cyclic voltammetry
Au - Gold	GFET – Graphene field-effect transistor
Boron – Br	H – Hydrogen
C – Carbon	HPLC – High pressure liquid chromatography
Ca – Calcium	INL – International Iberian Nanotechnology Laboratory
Cl – Chloride	K – Potassium
CSF – Cerebrospinal fluid	LOD – Limit of detection
CVD – Chemical vapor deposition	Mg – Magnesium
Cu – Copper	N - Nitrogen
Cr – Chromium	Na – Sodium
DAC – Digital-to-analog converter	Ni – Nickel
DDT – 1-Dodecanethiol	NHS – N-Hydroxysuccinimide
DOS – Density of states	PBS – Phosphate buffered saline
DMF – N, N-Dimethylformamide	PCB – Printed circuit board
EDL – Electrical double layer	PET – Positron emission tomography
EFO – Electrical flame-off	PMMA – Poly(methyl methacrylate)
EG-GFET – Electrolyte-gated graphene field-effect transistor	PBSE – 1-Pyrenebutanoic acid N-hydroxy-succinimide ester
ELISA – Enzyme-linked immunosorbent assay	
ETA – Ethanolamine	
Fe – Iron	

SELEX – Systematic evolution of ligands by
exponential enrichment

Si – Silicon

Ti - Titanium

Tween 20 – Polysorbate 20

US – Ultrasound

VMAT – Vesicular monoamine transporter

W - Tungsten

XPS – X-Ray Photoelectron Spectroscopy

λ_D – Debye length

1. INTRODUCTION

Neurons, the principal cells in the brain, use electrical impulses and chemical messengers to communicate with each other, encoding and processing complex information from the external world. Although technology to record and analyze electrical activity of the brain has been fast evolving over the past 60 years, the monitoring of neurotransmitters, the chemical messengers in the brain, remains a challenge and is an expanding area of active research [1]. Neurotransmitters are present in the nervous system at very low concentrations, they are responsible for proper brain function and their dysfunction underlies mental disorders. Being mixed with many other biochemical molecules and minerals makes their selective detection and measurement difficult. The precise detection of neurotransmitters and their dynamics would allow a better understanding of the brain encodes and processes information and ultimately of brain disorders, and the development of more efficient diagnostics and therapeutics.

The first technologies used to detect neurotransmitters involved brain microdialysis, mass spectroscopy, high pressure liquid chromatography (HPLC), and capillary electrophoresis and were later replaced with imaging techniques, proton nuclear magnetic resonance and magnetic resonance imaging [4].

Recent approaches to measure neurotransmitters include biosensors and microelectrodes. However, due to brain's complexity there are still many challenges like working with complex samples, obtaining good temporal resolution, and working at miniaturized sample preparations such as single neurons. Thus, for accurate detection and monitoring, a neurotransmitter has to be selectively identified among other biomolecules and other neurotransmitters ideally with a miniaturized and localized sensor.

Dopamine is a neurotransmitter with critical roles in the human brain and body, and its dysfunction underlies several brain disorders. Dopamine's function is crucial in the central nervous, renal, cardiovascular, and hormonal systems. High dopamine level indicates cardiotoxicity leading to rapid heart rates, hypertension, heart failure, and drug addiction [20]. However, low dopamine levels can cause stress, Parkinson's disease [19], Alzheimer's disease [21], and depression [30]. Despite dopamine's critical roles, current sensors for its detection typically lack relevant selectivity or sensitivity, or are incapable of being miniaturized for localized detection, which has been hindering the development of reliable diagnostics and potentiating earlier and more efficient treatment for dopamine-related disorders.

The most frequently used dopamine biosensors are electrochemical because dopamine being an electroactive molecule can be easily oxidized without an enzyme, thus it can be simply measured using electrochemistry. Nevertheless, this means that dopamine is suffering a redox reaction and similar oxidation signals can be acquired from dopamine interferents and analogues in biological samples or tissues, meaning that these sensors lack the desired selectivity [1]. Dopamine detection through imaging techniques indirectly measure dopamine release with high spatiotemporal resolution but require the use of small identifier molecules (labels), such as fluorescent dyes which poses translational problems. Microdialysis combined with high-performance liquid chromatography is also an approach able to detect extracellular dopamine but lacks spatiotemporal resolution [27]. Different biosensors with enzymes and antibodies were also reported but such molecules lack stability, and present poor limits of detection.

Aptamer-based sensors which include oligonucleotides or peptide molecules that are synthetically designed to bind to a specific target molecule as the recognition element, are a novel approach which has remarkable flexibility and convenient design of their structures has led to novel biosensors that have exhibited high sensitivity and selectivity [23].

To possibly overcome current limits and advance the state-of-the-art of dopamine detection, we engineered a novel solution combining a dopamine-specific DNA aptamer and graphene field-effect transistors.

Graphene is a monolayer of carbon arranged in a hexagonal lattice. Graphene and graphene-based materials are being increasingly used in a wide range of fields from electronics to biomedical applications due to their unique properties. These properties vary from lightness, great flexibility, high thermo-electrical conductivity, low noise, optical transparency, and biocompatibility to functionalization. Making it an outstanding material for sensors, overcoming the flaws of silicon biological and chemical sensors like, wafer production that relies on toxic products which reduces their intrinsic biomedical applications, silicon's reduced biocompatibility and small sensing surface area which hinders its performance in terms of efficiency and sensitivity. Another major disadvantage of silicon-based devices is their temperature dependence which makes them very limited when used for *in vivo* monitoring [73].

Graphene-based biosensors have been used for the detection of biologically relevant molecules such as glucose, DNA, or cholesterol [1] and are an emerging approach for brain sensing. This 2D material can be functionalized to detect very low concentrated analytes due to its one atom

thickness and excellent carrier mobility. Its non-toxicity, stability (due to strong covalent bonds in plane) and biocompatibility also make it a good contender for *in vivo* experiments in brain cells.

A type of graphene-based biosensor is the graphene field-effect transistor (GFET). This device is based on the typical field-effect transistor effect where a gate contact modulates the electronic conductance of the graphene channel connecting the source and drain. Additionally, for biosensing, the graphene channel can be modified with an immobilized biorecognition probe for the target of interest. The EG-GFET is a particular class of GFETs that uses an electrolytic solution as the gate dielectric. This liquid medium is convenient for bringing the molecules into contact with graphene while operating the GFET at very low voltages (1 V max) thanks to the enormous capacitance resulting from the very thin electrical double layer (EDL) that form at the graphene-liquid interface.

We used electrolyte-gated graphene field-effect transistors fabricated at the International Iberian Nanotechnology Laboratory (INL) and functionalized graphene's channel with a dopamine-specific DNA aptamer probe. When dopamine binds to the aptamer on the graphene surface, the electronic charge redistribution changes the electric field at the graphene surface, resulting in 'local gating', i.e., in the local modulation of the electrostatic gate field. Local gating alters the electronic conductivity in the channel and the overall device response, which in graphene transistors is a "V"-shaped curve whose minimum is abusively called the Dirac point. The name stems from a singularity in the graphene electronic density of states (DOS), called the Dirac point, located at the point where the valence and conduction bands meet. At the Dirac point, the DOS is null, and therefore, the conductivity has a minimum when the Fermi level is close to that energy. In the EG-GFET biosensor, the transistor Dirac point shift, induced by local gating, is proportional to the concentration of the target molecules bound to the immobilized probes.

Since dopamine detection with this aptamer by a graphene transistor has never been reported before, a graphene functionalization method had to be developed and assessed. After that, dopamine detection experiments were carried out *in vitro* in electrolyte buffers and in *ex vivo* samples from mice models. Dopamine detection experiments *in vitro* were carried out in hundreds of sensors (transistors) and the lowest limit of detection ever reported for dopamine was obtained, at attomolar concentration, in phosphate buffered saline (PBS) and artificial cerebrospinal fluid (aCSF). Additionally, dopamine was also detected with great sensitivity in *ex vivo* experiments with cerebrospinal fluid and brain striatum homogenate samples extracted from mice.

Combining these two novel methods, target recognition by aptamers with graphene sensing could allow new limits of detection to be reached for dopamine, and therefore potentiate better understanding of how this neurotransmitter works in the brain.

This thesis is organized as follows. In chapter 2, a review of neurotransmission, the roles of dopamine in the brain and methodologies for dopamine biosensing are presented. In chapter 3, a review of graphene, graphene field-effect transistors and their biosensing state of the art can be found. In chapter 4, the fabrication, assessment and characterization of our electrolyte-gated graphene field-effect transistors is described. In chapter 5, our surface functionalization and passivation method is presented as well as its characterization by spectroscopy techniques. In chapter 6, *in vitro* experiments for dopamine detection with our sensors in different electrolytes are described, as well as target selectivity experiments with dopamine analogues and interfering agents. In chapter 7, *ex vivo* experiments with dopamine detection in biological samples are described.

2. NEUROTRANSMITTER'S BIOSENSING

A neuron is an electrically excitable cell that communicates with other cells through specialized connections, synapses. It is the functional and structural unit of our brain and the entire nervous system and consists of a cell body (soma), dendrites, and axon.

Figure 1 shows an exemplificative illustration of a neuron's structure.

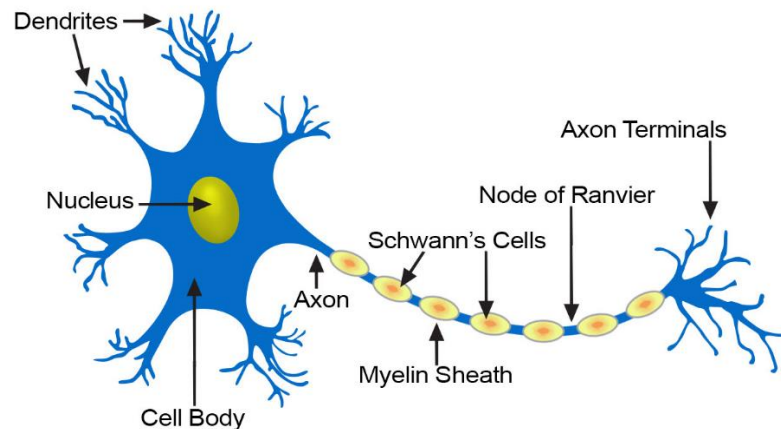


Figure 1. Exemplificative illustration of a neuron cell [30].

The soma is localized at the center of the neuron, it contains its nucleus and is where protein synthesis, and metabolic activity takes place. Dendrites are the numerous extensions in form of small branches from the neural body which relay inputs from other neurons to the soma. The axon is the main prolongation of the neuron, and it transports neuronal signals away from the soma to other neurons. The axon terminates in axonal terminals which contact the dendrites of other neurons.

Neurons communicate with each other via electrical events called action potentials and chemical molecules called neurotransmitters. At the junction between two neurons, lies the synapse, a specialized structure, where the axon terminal from one neuron (pre-synaptic neuron) contacts with a dendrite of another neuron (post-synaptic neuron).

Neurotransmission is the process by which neurotransmitters are released by the axon terminal of the presynaptic neuron and bind to and react with the receptors on the dendrites of the postsynaptic neuron. Neurotransmitters are released from synaptic vesicles, by incoming action potentials generated at the soma, into the synaptic cleft where they are received by neurotransmitter receptors on the target cell. The neurotransmitter can either excite or inhibit the other neuron from triggering its own action potential. Figure 2 shows a neurotransmission event.

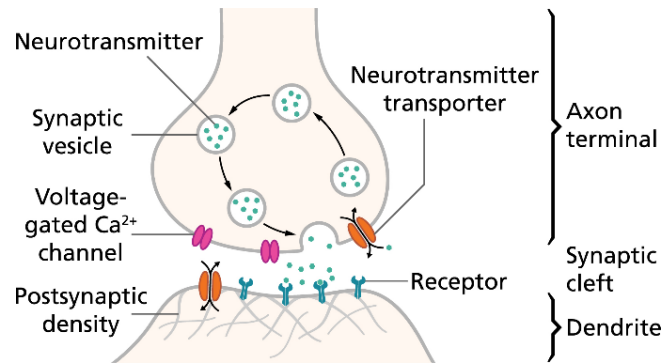


Figure 2. Exemplificative illustration of a neurotransmission event. The presynaptic neuron (top) releases a neurotransmitter, which activates receptors on the nearby postsynaptic cell (bottom) [29].

A neurotransmitter is a signaling molecule secreted by a neuron and its purpose is to affect another cell through a synapse. To be considered a neurotransmitter a substance has to be synthesized in the neuron, be found in the presynaptic end and released to have an effect in the postsynaptic cell, then be imitated by exogenous application to the postsynaptic cell and have a specific mechanism for termination of its action [31]. As such, more than 100 neurotransmitters have been identified and can be categorized according to varying criteria.

One exemplificative classification divides neurotransmitters in different categories like monoamines (e.g. dopamine, norepinephrine, epinephrine, histamine, serotonin), amino acids (e.g. glutamate, aspartate, D-serine, gamma-Aminobutyric acid) and peptides (e.g. oxytocin, somatostatin). A single neuron can synthesize and release different types of neurotransmitters and can contain receptors for several types of neurotransmitters. This creates a complex environment of chemical communication in the brain, critical for the encoding and processing of information.

Many neurotransmitters are synthesized from simple and plentiful precursors such as amino acids, which are readily available and only require a small number of biosynthetic steps for conversion [3]. Because neurotransmitters play a fundamental role in neurotransmission, their dysfunction underlies a wide range of mental disorders including depression, drug dependence, schizophrenia, and degenerative diseases among many others [2]. Thus, the development of novel methods to detect neurotransmitters in the brain is paramount to the development of more efficient diagnostics and therapeutics for brain disorders.

2.1. Dopamine

Dopamine, 3,4-dihydroxyphenethylamine, is a neurotransmitter with critical roles in the human brain and body. It is part of the monoamine neurotransmitters, and in particular of the catecholamine group, because it consists of a catechol structure (a benzene ring with two hydroxyl side groups) with one amine group attached via an ethyl chain. As such, dopamine is the simplest possible catecholamine. In fact, dopamine constitutes about 80% of the catecholamine content in the brain [32].

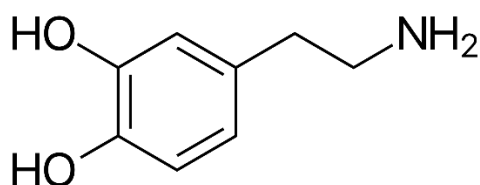


Figure 3. Dopamine's chemical structure.

Dopamine function is crucial in the central nervous, renal, cardiovascular, and hormonal systems. In blood vessels, it inhibits norepinephrine release and acts as a vasodilator; in the kidneys, it increases sodium excretion and urine output; in the pancreas, it reduces insulin production; in the digestive system, it reduces gastrointestinal motility and protects intestinal mucosa; and in the immune system, it reduces the activity of lymphocytes. With the exception of blood vessels, dopamine is synthesized locally and exerts its effects near the cells that release it. A significant amount of dopamine circulates in the bloodstream, mostly in the form of dopamine sulphate, but its functions there are not entirely clear [5].

Since dopamine is incapable of crossing the blood-brain barrier, it must therefore be synthesized inside the brain to perform its neuronal activity. Dopamine synthesis pathways involves several molecules. The main pathway for dopamine synthesis is through tyrosine, a non-essential amino acid, which is transported across the blood brain barrier. L-Tyrosine is converted into L-3,4-dihydroxyphenylalanine (L-Dopa) by tyrosine hydroxylase. The amino acid L-Dopa is the direct precursor of dopamine because it can be converted into dopamine with help of the aromatic amino acid decarboxylase, by removing the carboxyl group [25].

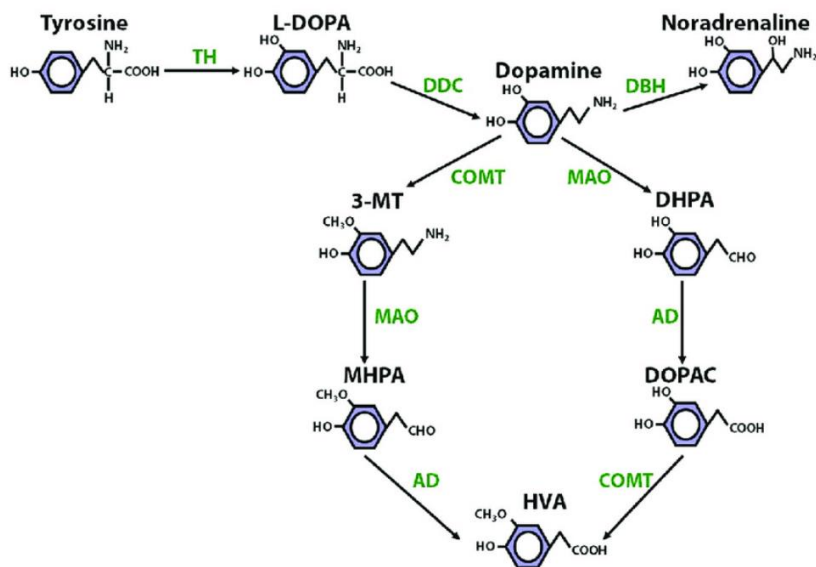


Figure 4. Dopamine's synthesis pathways [34]. Homovanillic Acid (HVA); Tyrosine Hydroxylase (TH); Dopamine β-Hydroxylase (DBH); Monoamine Oxidase (MAO); Aldehyde Dehydrogenase (AH); Dopamine Decarboxylase (DDC); 3-Methoxytyramine (3-MT); 3,4-Dihydroxyphenylacetaldehyde (DHPA); 3-Methoxy-4-Hydroxyphenylacetaldehyde (MHPA).

Dopamine is broken down into inactive metabolites by a set of enzymes, monoamine oxidase, catechol-O-methyl transferase, and aldehyde dehydrogenase, acting in sequence. Different breakdown pathways exist but the main end-product is Homovanillic acid, which has no known biological activity [5].

The brain includes several distinct dopamine pathways. One example is the mesolimbic pathway which transmits dopamine from the ventral tegmental area – the source of most dopaminergic neurons in the brain – to the striatum and plays a major role in the motivational component of reward-motivated behavior [32].

In this pathway, positive events and stimuli increase the levels of dopamine in the striatum, as well as the anticipation of this events and stimuli [32]. Thus, disruption of dopamine transmission in this pathway can lead to attention deficit disorder [33], schizophrenia [20] and substance addition [27].

Another important brain dopamine pathway is the nigrostriatal pathway which transmits dopamine from the substantia nigra pars compacta to the striatum and is involved in motor control [32]. Dysfunction of this pathway can lead to Parkinson's disease and other motor related disorders [27].

Despite dopamine's critical roles, current sensors for its detection typically lack relevant selectivity or sensitivity, or are incapable of being miniaturized for localized detection, which has

been hindering the development of reliable diagnostics and potentiating earlier and more efficient treatment for dopamine-related disorders.

2.2. Dopamine detection state of the art and detection issues

Neurotransmitters are present in the nervous system at very low concentrations, and they are mixed with many other biochemical molecules and minerals, thus making their selective detection and measurement difficult. Measuring neurotransmitters *in vivo* is only possible on the extracellular space of the central nervous system and to do so stable and accurate measurements with high selectivity and selectivity are required. Neurotransmitter content and release are also studied *in vitro* through analysis of cells in culture and *ex vivo* tissue preparations such as brain slices. Although numerous techniques to do so have been proposed in the literature, neurotransmitter monitoring in the brain is still a challenge and the subject of ongoing research [1].

The most recent approaches to detect dopamine include biosensors and microelectrodes. However, there are still many challenges like working with complex samples (neurotransmitters can be mixed with other molecules), obtaining good temporal resolution, and working at miniaturized sample preparations such as single neurons. Thus, for accurate detection and monitoring, a neurotransmitter has to be localized and identified among other biomolecules and other neurotransmitters, as they can have similar behaviors.

Earlier methods for dopamine detection included brain microdialysis, followed by HPLC, mass spectroscopy, and capillary electrophoresis. Extracellular brain fluid can be extracted by microdialysis through a probe implanted in the brain and the analytes collected over time can be analyzed by quantitative techniques. HPLC is a procedure for separating components from a mixture of chemical substances by pumping a pressurized liquid solvent, containing the sample mixture, through a column filled with a solid adsorbent material. Sample's components interact differently with the adsorbent material, creating different flow rates for the different components which leads to the separation of the components as they flow out of the column [36]. Analytes separated in the column can then be analyzed by mass spectroscopy which is an analytical technique that is used to measure the mass-to-charge ratio of one or more molecules present in a sample [37]. This technique has been used extensively to study reward-mediated behaviors in

mice, such as drug addiction, and demonstrated an increase in extracellular dopamine but has poor spatiotemporal resolution [27].

Other earlier methods for dopamine detection included imaging techniques such as functional magnetic resonance imaging and [4] positron emission tomography (PET) [35]. It operates with positron emitting ligands that target specific receptors to supervise dopaminergic activity. Its diverse set of ligands and high sensitivity allow it to be used in humans. But the disadvantages surpass the advantages, ligands are expensive, difficult to synthesize, and can have short half-lives; poor spatiotemporal resolution makes it impossible to distinguish ligand signal from its metabolite signal. Functional magnetic resonance imaging is non-invasive and can be used in humans. Ligands can be used to increase spatiotemporal resolution or highlight specific structures; however, information is largely structural and not functional [27].

Dopamine indirect imaging techniques involve fluorescent sensors that either co-transit with dopamine through the synaptic vesicle cycle or reversibly bind to dopamine resulting in a change in fluorescence. These methods offer spatial resolutions that provide synapse- and circuit-level detail.

Fluorescent microscopy methods for dopamine detection are based on small-molecule- or protein-based sensors that indirectly measure dopamine release with high spatiotemporal resolution. There is a diverse set of fluorescent dyes and protein sensors but they have low bio-penetrance and small wavelengths, can be phototoxic and have limited use *in vivo* [27]. In two-photon microscopy, fluorescent sensors are imaged using two-photon excitation. However *in vivo* imaging is expensive and difficult to implement [27]. Fluorescent imaging techniques are not practical for human application due to the extent of dopamine neurons within the brain, the limited bio-penetrance of ultraviolet and visible light and, since the brain doesn't work in the optical domain, to do so it is needed to deliver fluorescent molecules, and such is not allowed for humans.

The most frequently used dopamine biosensors are electrochemical because dopamine being an electroactive molecule can be easily oxidized without an enzyme, thus it can be simply measured using electrochemistry. The sensitivity and selectivity for dopamine can be enhanced by modification of the electrode with nanomaterials and affinity ligands [10]. For example, fast-scan cyclic voltammetry (FSCV) was the main neurotransmitters detection method during many years that allowed us to better understand dopamine's functions in distinct sub-regions of the striatum. It is based on fast waveform scans from -0.4 V to $+1.3$ V at 400 V/s, repeated at 100 ms intervals applied to a microelectrode to quickly oxidize and reduce electroactive species at the electrode's

surface [22]. This rapid scan produces a current that appears from the charging of the electrical double layer and is proportional to the capacitance of the electrode. Considering that different molecules have different oxidation and reduction peaks, the peak potentials can provide a chemical signature to identify the molecules detected. FSCV offers a vast improvement in temporal resolution compared to microdialysis due to its ability to measure extracellular concentrations as low as 50 nM [27].

Other voltametric methods, that rely on measuring the current flowing between two electrodes, a working electrode and a counter electrode, subjected to a voltage difference during a redox chemical reaction, were also used to detect dopamine with varying success and limits of detection (LOD)s [22]. Nevertheless, although voltametric methods can achieve high temporal resolution, their main disadvantage is that similar molecules have similar oxidation peaks. This means dopamine precursors, analogues and other biological interferents may be difficult to dissociate from dopamine leading to poor selectivity.

The most recent approaches to detect dopamine include biosensors and microelectrodes. Typically, a biosensor contains a biological molecular probe for target identification like antibodies, enzymes, or aptamers. The biomolecule is coupled to a signal transduction system to indicate the specific binding event.

Enzymes are commonly used for sensing small molecular metabolites since they have high catalytic efficiency and good specificity, and can quickly convert substrates into products. For the detection of dopamine, many enzymes have been used including polyphenol oxidase, horseradish peroxidase, laccase, and tyrosinase. Polyphenol oxidase biosensor has a limit of detection (LOD) of 50 nM [6], horseradish peroxidase LOD is around 600 nM [7], Laccase LOD is 29 nM [8] and tyrosinase LOD is 1 nM [9]. Enzymes-based dopamine's limit of detection is on the nanomolar level.

Antibody-based biosensors offer many advantages like high binding affinity, greater specificity, and well-developed detection platforms such as enzyme-linked immunosorbent assay (ELISA) and lateral flow assay devices (FLA) [39]. However, antibodies suffer from instability and batch-to-batch variation since antibody production often requires animals [10]. Dopamine antibodies were immobilized in gold nanoparticles and signals were measured with localized surface plasmon resonance giving a limit of detection also on the nanomolar concentration [11]. However, direct adsorption of dopamine on these nanorods cannot be ruled out since dopamine is known to adsorb on both gold and silver surfaces. And such method has not been used on real

samples. Namkung *et al.* established an indirect competitive immunoassay to detect dopamine. This method was effective for the detection of dopamine in urine but was not sensitive enough at low concentrations ($<0.1 \mu\text{M}$). Therefore, this method was not used for serum samples due to the expected low concentration of dopamine in serum [10].

Aptamers are oligonucleotide (DNA, RNA or XNA) or peptide molecules that are synthetically designed to bind to a specific target molecule. An aptamer binds to its designated target through different non-covalent interactions (e.g. electrostatic interactions, hydrophobic interactions, or induced fitting) [38]. Their applications vary from research, industrial and to clinical purposes as macromolecular drugs [38]. Nucleic acid aptamers are generated via *in vitro* selection from an initial large random sequence pool or through systematic evolution of ligands by exponential enrichment (SELEX) [24].

RNA aptamers form diverse three-dimensional structures, enabling a larger number of conformations when compared with DNA aptamers [25], and can form smaller structures than DNA aptamers of the same number of oligonucleotides. However, DNA aptamers are more stable than RNA aptamers which have intrinsic chemical instability *in vitro* due to a 2' hydroxyl group [26].

Aptamer-based sensors offer great specificity and high affinity since they are single stranded nucleic acids that bind specifically to their target. Not only are they smaller than antibodies but they also change their conformation which can potentiate faster detection. Nucleic acids have programmable and predictable secondary structures, allowing versatile biosensor designs. Aptamers are particularly attractive for binding small molecules [12], whereas antibodies have more difficulty for these targets [10]. Their remarkable flexibility and convenient design of their structures has led to novel biosensors that have exhibited high sensitivity and selectivity [23].

The first dopamine aptamer was an RNA isolated by Mannironi *et al.* in 1997 [13]. Later, this aptamer was converted into a DNA aptamer which was tested in several biosensors and, finally, the reported limit of detection for dopamine increased from nanomolar to picomolar [14]. Recently, a new dopamine DNA aptamer has been reported by Nakatsuka *et al.* [15]. The new aptamer is not only smaller than the one previously reported, from 57-mer to 44-mer, but also was applied on field-effect transistors which allowed the limit of detection for dopamine to decrease to the femtomolar concentration range [15]. Target-induced conformational changes of the negatively charged aptamer in close proximity to semiconductor channels regulated the conductance in physiological buffers, resulting in highly sensitive and selective detection.

Many aptamer-based biosensors have been reported with different transduction mechanisms proving the versatility of the aptamers. Fluorescent biosensors are frequently used because of their fast response, convenient operation, and high limit of detection (picomolar); however, they are not label-free since they use quenchers to mediate the photon-emission. Colorimetric biosensors were also studied, Zheng *et al.* developed a colorimetric biosensor using unmodified gold nanoparticles because the authors believed that dopamine binding can fold the RNA-derived DNA aptamer and prevent aptamer adsorption on the gold nanoparticles, however, the limit of detection was only of 0.3 μM [16]. Besides the fluorescent and colorimetric biosensors, many other aptamer-based biosensors have also been reported based on personal glucose meters, surface enhanced Raman spectroscopy, and surface plasmon resonance [10].

Most aptamer-based electrochemical biosensors are made up of three parts consisting of a DNA aptamer for dopamine, a gold or glassy carbon electrode, and a nanomaterial or nanocomposite to enhance sensitivity and conductivity. The role of aptamers is to selectively recruit dopamine to the electrode surface, while the signal is still obtained from the redox chemistry of dopamine [10].

Li *et al.* developed a nanoelectronic biosensor by modifying DNA aptamers on a multiple-parallel-connected silicon nanowire field-effect transistor (FET). FETs work by measuring the current passing through a transistor, in this case, the current was modulated by aptamer binding [17]. The sensor had good specificity and was applied in real time sensing however, the LOD was 0.01 nM.

Although RNA is less stable, the use of RNA aptamers was still explored. Farjami *et al.* reported an electrochemical RNA aptamer-based biosensor resistant to interference from other neurotransmitters [18]. The sensor was based in amperometry, it applies a fixed potential to a working electrode and measures the resulting oxide-reduction reaction current, which is proportional to the analyte concentration. Like voltammetry, the current through the working electrode is measured with respect to a reference electrode, but the temporal resolution of amperometry is not limited to the duration of the cycle. Thus, amperometry is more suitable to analyse the kinetics of neurotransmitter release. The amperometric signal was related with concentration of dopamine between 100 nM and 5 μM , the LOD was not low enough and nonspecific adsorption of biologic fluids affected the measurements.

Split aptamers are composed of two or more DNA strands that mimic segments of a larger parent aptamer. Nucleic acid aptamers are split into two fragments that could specifically form a ternary assembly in the presence of ligand improving the sensor's specificity and sensitivity in

presence of low concentrated analytes [40]. Despite being very difficult to engineer aptamers into split aptamers, Liang, Yuanying, *et al.* reported a split aptamer that was functionalized on an organic electrochemical transistor. Dopamine binding was detected with a LOD of 0.5 fM, which is currently the lowest LOD reported for dopamine [28].

Antibody-based biosensors lack fast and sensitive detection needed for *in situ* assays; enzyme-based biosensors are less stable and have complex immobilization processes, and nanomaterial-based sensors rely on dopamine's oxidation potential for detection which lacks sensitivity because of other redox molecules' similar oxidation signals.

Fluorescent methods provide synaptic-level measurements but are not adaptable to humans. PET offers high sensitivity but lacks temporal-spatial resolution and require ligands with short half-lives. Magnetic resonance imaging techniques have good selectivity but are less sensitive than PET or fluorescence, and not yet adjusted for clinical use [27].

In conclusion, DNA aptamers possess many advantages including versatile signal transduction mechanism, fast response time, high stability, low cost and easy modification. Most common types of reported aptamer biosensors were optical and electrochemical.

The complex medium that is the *in vivo* environment requires biosensors to be specific to the neurotransmitter in question, to understand the biochemical and physiological roles of dopamine, in this case, under various stimuli. Therefore, using DNA aptamers coupled to a signal transduction system is a promising approach for label-free dopamine detection *in vivo*.

To go beyond the current state of the art limit of detection for dopamine, currently at the femtomolar concentration range, we combined a DNA aptamer for highly selective target recognition with field-effect transistor with a graphene channel, a novel two-dimensional material as an active surface electrode. Because of graphene's exceptional conducting properties, we hypothesized that our new sensors based on electrolyte-gated field-effect transistor functionalized with a DNA aptamer could go beyond the currently reported limit of detection for dopamine. The next chapter introduces graphene field-effect transistors and their application in biosensing devices.

3. GRAPHENE FIELD-EFFECT TRANSISTORS

3.1. Graphene

Graphene is a two-dimensional crystalline material that Geim and Nosolesov first isolated in 2004 [41]. Graphene is a monolayer of carbon atoms hybridized sp^2 arranged in honeycomb lattice with a thickness of $3 \text{ \AA}$ – the size of a carbon $2p$ -orbital – which is twice the carbon-carbon bond length. Figure 5 shows a picture of graphene and a ball-and-stick representation of its carbon hexagonal lattice.

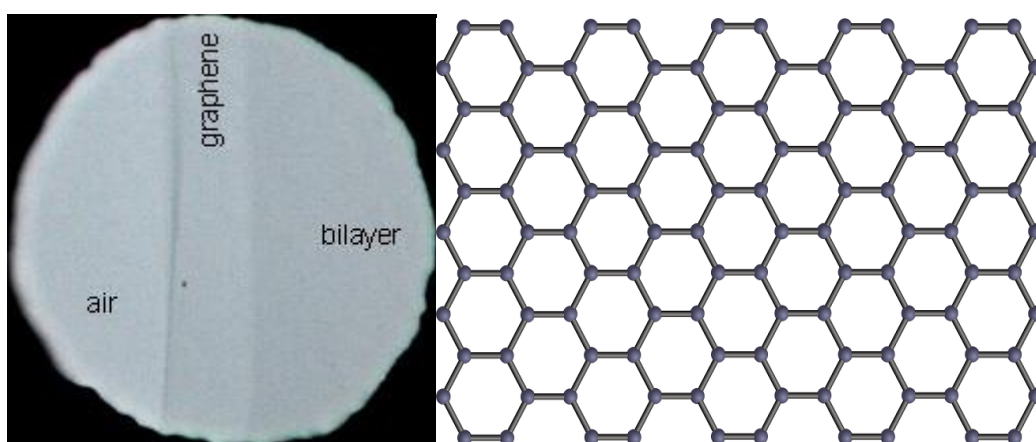


Figure 5. Picture of monolayer and bilayer graphene (left) [67], and representative figure of graphene (right).

The in-plane bonds between the carbon atoms in the lattice atoms are covalent σ -bonds involving the three electrons occupying the sp^2 orbitals. The p -orbital that does not hybridize is oriented perpendicularly to the lattice [41]. P -orbitals laterally overlap to form a network of covalent π bonds highly conjugated, occupied by high-mobility electrons – one per atom – is mainly responsible for the unique graphene electronic properties and the interaction with the surrounding environment.

Graphene sheets can pile up in stacks with few or many layers, like in graphite, where π - π interactions supply the van der Waals interlayer forces that stabilize the stacks. Because of the weak π - π non-covalent interaction, the separation of layers is easy, which led to the first technique used for graphene production, mechanical exfoliation. It refers to a group of top-down techniques that exfoliate a bulk van der Waals solid into its corresponding isolated layers by applying energy to disrupt the weak inter-layer bonds [43]. A variation of the technique, known as scotch tape

exfoliation, became famous for its simplicity, low cost, low-technology level, and for being used by the 2014 Nobel prize winners in preparing their samples for fundamental studies at Manchester University. The scotch tape method consists of rubbing a graphite crystal against a scotch tape, then releasing the resulting flakes of micro- and nanometric lateral dimensions onto the surface of a substrate randomly.

Besides mechanical exfoliation, graphene production has been carried out by liquid-phase exfoliation using shear exfoliation approaches [42], such as high shear mixing or wet jet milling splitting. Apart from these top-down techniques, a vast effort has been dedicated to developing bottom-up graphene growth, with atomic control of the growth process and the resulting graphene structure. Most important among these techniques, due to the high electronic quality of the resulting material, comparable to that of mechanically exfoliated flakes, are epitaxial growth and chemical vapor deposition (CVD) [69].

In this work, graphene is grown in a CVD system with a 5" diameter quartz process tube with a load lock. The tube accommodates substrates measuring up to 10 cm × 15 cm (see chapter 4).

Graphene is a semi-metal or a zero-bandgap semiconductor because its electronic density of states (DOS) has a singularity where the conduction and valence bands meet, the so-called Dirac point. In the vicinity of the Dirac point, a linear dispersion of the electron energy-momentum relation is observed, going almost symmetrically towards the valence and conduction bands, forming the so-called Dirac cones [45]. These characteristics are a consequence of the relativistic nature of the electrons in graphene, close to the Dirac points Figure 6 shows graphene's electronic band structure and Dirac cones at the edge of the six-fold symmetry Brillouin zone (zone defined by the primitive cell in reciprocal space of the crystal lattice).

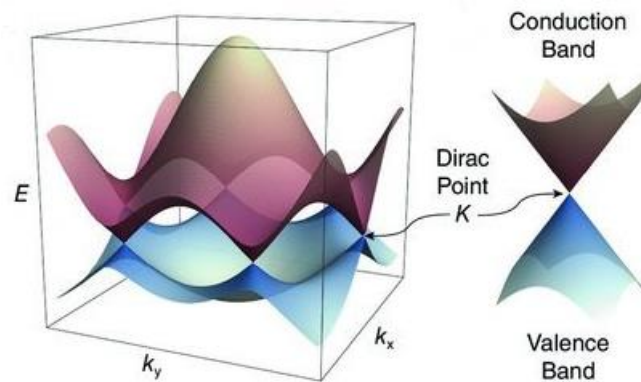


Figure 6. Electronic band structure of graphene (left) and Dirac cones intersection at the Dirac point [46].

The Fermi energy, or Fermi level, E_F (defined as the energy difference between the highest and lowest occupied single-particle states in a quantum system of non-interacting fermions at absolute zero temperature) [70] for intrinsic graphene is at the Dirac energy, i.e., the energy of the Dirac point. The Fermi energy can be considerably different from the Dirac energy in doped graphene devices. Since there is no band-gap, the Fermi level can move continuously between bands, which has significant consequences in the characteristics of graphene field-effect transistors, as discussed in the next section.

After the clean-room fabrication process, the graphene channel in the GFETs is p-doped. As discussed in the following chapters, the doping level changes as different materials contact graphene throughout the work.

Because of its unique band structure, graphene displays novel transport effects such as minimum conductivity and ambipolar field-effect transport, where carriers can be changed from electrons to holes and vice-versa by gating with an electric field, a possibility absent in most materials [41]. Graphene's carrier mobility, as high as $10^5 \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$, is the largest ever reported for semiconductors or semimetals [46]. Even at the highest electric-field-induced carrier concentrations, mobility remains high and is barely affected by chemical doping [71].

In polycrystalline graphene deposited by CVD, the carrier mobility at room temperature is lower, typically in the range $\sim 10^3\text{-}10^4 \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$. In the CVD graphene used in this work, the measured carrier mobility was in the range $2000\text{-}6000 \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$.

3.2. Graphene field-effect transistor

The field-effect transistor (FET) is a three-terminal (gate, source, and drain) device where a gate voltage (V_{GS}) controls the channel's conductance between source and drain. There is no current flow between gate and source, possibly apart from a small leakage current [74]. In the saturation regime, the source-drain current (I_{DS}) is independent of the source-drain voltage (V_{DS}) and only depends on V_{GS} (figure 6). This regime is unattainable by the graphene FET, which never saturates because graphene is a zero bandgap semiconductor. To explain why it is necessary to recall that V_{GS} modulation of the channel conductance is achieved by moving the Fermi level across the DOS of the channel material. In semiconductors, once V_{GS} is large enough to place E_F in the semiconductor forbidden energy bandgap, then there are no new states available for electron transport and I_{DS} saturates (apart from second-order effects that we do not discuss here). In

graphene, E_F moves continuously between the valence and conduction bands, and therefore the GFET never saturates, while it also never turns OFF (figure 7).

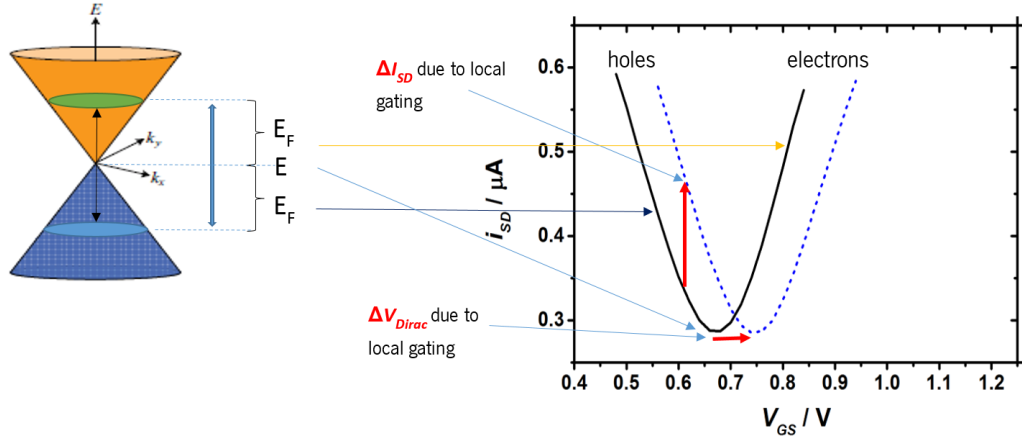


Figure 7. GFET's DOS and transfer curve. Dirac cones (left) and graphene transfer curve (right).

When E_F moves from the valence to the conduction band through the Dirac point – where the DOS is null – the carrier concentration derivative with respect to V_{GS} changes sign but is never zero. Therefore, I_{DS} exhibits a minimum when E_F is at the Dirac point and increases again as E_F penetrates the next band (the effect is symmetric and is observed when moving from valence to conduction band and vice-versa). Because of the linear energy-momentum dispersion relation for electrons occupying states close to the Fermi level, in the Dirac cones, the GFET transfer curve presents two linear symmetric branches relative to the point of the lowest channel conductance, also called the charge neutrality point (see figure 8, right).

For comparison, figure 8 shows the characteristic transfer curve (I_{DS} as a function of V_{GS}) for a silicon field-effect transistor.

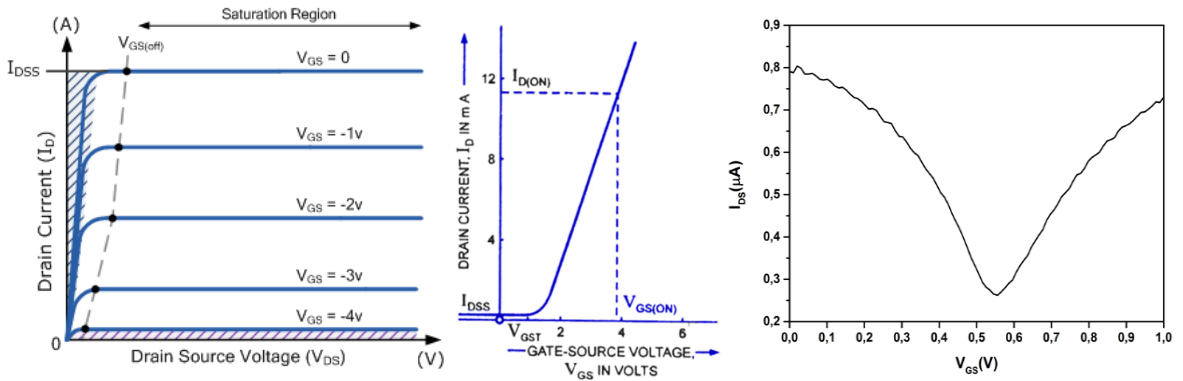


Figure 8. FET's V_{DS} as function of I_{DS} curve (left), FET's V_{DS} as function of I_D curve (middle) and GFET's V_{DS} as function of I_{DS} (right).

3.3. Electrolyte-gated graphene field-effect transistor

Like its 3D semiconductor counterpart, the graphene field-effect transistor (GFET) can be made in the top and bottom gate geometries. Figure 9 is an exemplificative representation of a GFET.

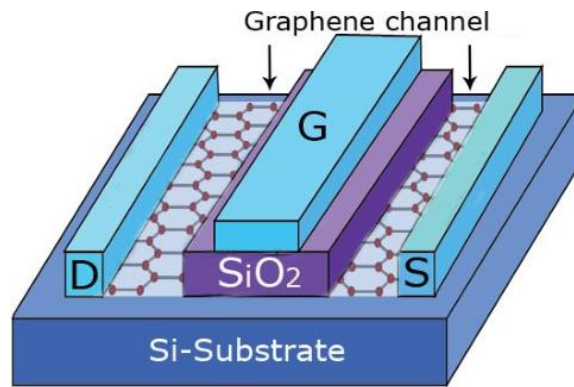


Figure 9. Exemplificative representation of a graphene field-effect transistor [68], drain (D), source (S) and gate (G).

Bulk semiconductors such as silicon, used in standard FETs are three-dimensional, while the FET conduction channel is 2D and forms at the semiconductor surface. The surface of a 3D material is a locus of lattice reconstruction and defects, namely dangling bonds, because the surface atoms have a very different neighborhood than bulk atoms. Consequently, 3D semiconductors need a passivated interface to form the transistor channel. This passivation is obtained by covering the semiconductor surface with a dielectric material that, in most cases, acts as the gate dielectric. The quality of the interface Si/SiO₂ is one of the reasons why today's prevalent CMOS technology is based on silicon. Thus, surface defects, or dangling bonds, usually dominate in any non-passivated semiconductor surface, preventing a channel from forming for transistor action. In this context, the great advantage of graphene is that being only one atom thick, it suffers no surface reconstruction, having no or few dangling bonds. The GFET channel forms naturally only by placing graphene between the source and drain metallic contacts: a graphene layer is a channel. This property, allied with graphene's high chemical stability, allows operating the GFET in ambient conditions, with the channel exposed to the environment, which is a remarkable fact that we explore extensively in biosensing devices, as discussed in the following paragraphs.

Liquid electrolyte-gated graphene field-effect transistors (EG-GFETs) are a particular class of GFETs where a liquid electrolyte drop-casted on the transistors' surface is used as the gate dielectric. In this case, the gate capacitance is dramatically increased compared to a solid-dielectric

transistor because the gate voltage drop occurs only at the electrical double layers (EDLs) that form at the solid-liquid interfaces, namely the electrode-electrolyte and the graphene-electrolyte interfaces. The EDLs are one Debye length thick, a quantity that depends on the ionic concentration in the electrolyte, but in normal conditions, is of the order of 1 to 10 nm.

This huge capacitance allows operating the EG-GFET at very low gate voltage (< 1 V), which is essential in working with biomolecules that often possess secondary and tertiary complex structures, susceptible to electric fields and potentials. Moreover, since most biosensing is done in aqueous media, a low voltage is required to avoid water electrolysis (which starts nominally at 1.2 V).

Working with biomolecules in aqueous media, it is crucial to consider the Debye-Hückel screening phenomenon where the electrical charge of the molecules is screened by the electrolyte counter-ions (those of opposite charge signal), forming EDLs that are similar to those mentioned above for the solid-liquid interfaces. Again, there is a specific characteristic length scale of this screening given by the Debye length, the distance from a molecule/surface where the electrostatic force is significantly reduced (to $1/e$). Since the Debye length is dependent on the electrolyte's ionic concentration and valence, it is essential to consider which electrolyte solution to use because they affect the electrostatics of the medium and, therefore, the EG-GFET operation. Figure 10 is an exemplificative illustration of the EDL on a EG-GFET.

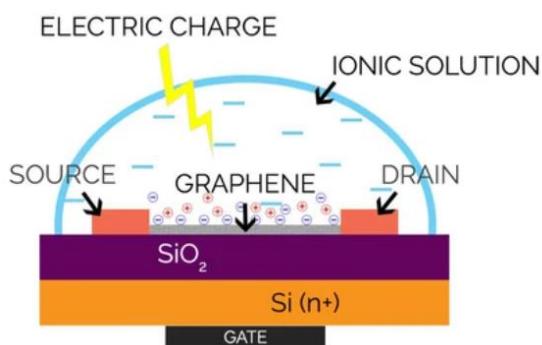


Figure 10. Exemplificative illustration of the electrical double layer (EDL) on a EG-GFET.

The EG-GFET biosensor transducing mechanism is local electrostatic gating. The graphene-electrolyte EDL is perturbed locally and changed by charged or polar species resulting from the biorecognition events between the probes immobilized on the graphene surface and the target biomolecules.

3.4. Graphene field-effect transistors' biosensing state of the art

Graphene's chemical and electronic properties make it an appealing option for constructing biosensing devices as a biorecognition and transduction platform [52].

Graphene-based devices can enable rapid, label-free, high-sensitivity sensors for healthcare point-of-care diagnostics and have the potential to replace other technologies that are high cost, require complex fabrication processes, have low sensitivity, low specificity and lack fast response required for *in situ* biosensing.

While graphene sensing and transducing capability are superlative, as discussed in the previous sections, it responds to any changes in its dielectric environment, and therefore is not selective for particular molecular targets. Thus, the graphene surface must be functionalized with molecular probes that render it target-selective for biosensing.

Generally, graphene does not bind with most materials. However, several surface chemistry strategies enable functionalization by forming binding sites on its surface. In these devices, graphene typically is exposed to permit functionalization of the channel surface and binding of receptor molecules.

Bioreceptors such as amino acids, antibodies, enzymes, or aptamers can be added through a linker molecule attached to the graphene surface. Molecules can attach to these sites through covalent bonding, electrostatic, or Van der Waals forces, modifying the graphene dielectric environment or, in some cases inducing charge transfer to graphene [44].

Recently, several well-controlled chemical functionalization procedures compatible with GFETs have been developed. GFETs have been functionalized with proteins, chemical compounds, and DNA molecules to make sensors for various applications.

Different chemistries appropriate for the functionalization of graphene devices were reported. They can be based on diazonium compounds that form a covalent bond to the graphene surface [56] or bifunctional pyrene compounds that interact with graphene through a π - π stacking interaction [57]. Due to the aromatic-ring containing amino acids on the surface, many proteins binding onto graphene surface by π - π stacking can be detected by doping effects.

The first significant study between chemically-modified graphene transistor and biomolecules was published by Mohanty and Berry in 2008 [58]. Their single-stranded DNA probes were anchored on the surface of the graphene sheet, followed by hybridization with complementary

DNA strands functionalized with fluorescence dyes. The hybridization process would affect the current and the electrical field.

Graphene has also been explored as a quencher for fluorescence-based detection. Chang *et al.* labelled an aptamer specific for thrombin with a fluorescent dye and used graphene as a substrate for non-specific adsorption achieving a LOD of 30 pM [53] by measuring fluorescence resonance energy transfer (FRET). Due to the non-covalent assembly between aptamer and graphene, fluorescence quenching of the dye takes place because of the FRET.

Electrochemical detection was also performed with graphene materials. For example, Viswanathan *et al.* [54] reported the use of graphene for enzymatic detection of glucose, based on the reaction between the enzyme and glucose, and achieved millimolar LOD. Despite having graphene as the recognition element, the low sensitivity of this method still prevailed and was caused by the lack of selectivity associated with the redox reactions [50].

In terms of electrical transduction platforms, Dong *et al.* reported a label-free use of graphene for electrical detection of single nucleotide mismatch in DNA with GFETs [55]. Graphene was patterned with gold nanoparticles that had the DNA probe attached to their surface, and when the complementary DNA was added, a shift to lower voltages of the Dirac point was observed at the lowest concentration of 100 nM.

Guo *et al.* described the fabrication of graphene transistors with conventional photolithographic patterning process. The resulting devices also show high sensitivity in label-free detection of DNA [59].

Zhu *et al.* explored thionine-GFETs, a DNA sensor decorated with amino-substituted oligonucleotide as DNA probe through the arm linker, acquiring an excellent linear response and a low detection limit of 0.1 pM [61].

Campos *et al.* recently reported an electrolyte-gated GFET capable of detecting DNA hybridization with superior sensitivity at attomolar label-free recognition [62]. This work utilized a similar graphene transistor to the one we introduce in chapter 4.

Ohno *et al.* produced label-free biosensors based on aptamer-modified GFETs for selective immunoglobulin E detection. In their experiment, the conductance of a GFET functionalized with immunoglobulin-specific aptamers decreased when the positively charged targets were introduced and bonded to the oppositely charged aptamers [60].

Her *et al.* detected alanine aminotransferase with a concentration range of 10 to 100 U/L using a graphene field-effect biosensor [63].

Compared to chemical molecules detection, detecting living cells faces more difficulties and challenges because of the complex interaction between graphene and living cell membranes. A biosensor based on GFETs functionalized with *E.coli* antibodies was successfully fabricated by Huang *et al.* The detection limit was 10 cfu/m [64]. Because GFETs are highly sensitive to pH, the metabolic activities of living cells could be revealed in real-time through current change since it is affected by the changes of the local pH due to the release of metabolic organic acids.

Ang *et al.* reported malaria-infected red blood cells detection with graphene-based transistors. The sensor array was integrated with microfluidics and achieved the single-cell flow sensing by executing a “flow-catch-release” [65].

An ultra-sensitive and flexible FET olfactory system was also demonstrated by Park *et al.* After O₂ or NH₃ plasma treatment to open the graphene bandgap, human olfactory receptors were bonded to amyl butyrate that was conjugated with a graphene platform. The results indicated that the sensitive sensor could achieve a LOD as low as 0.04 fM [66].

Graphene is also an emerging material for brain-sensing applications, and recent works have shown the use of graphene transistors for such applications. GFETs have been explored for detecting electroactive activity with a high signal-to-noise ratio from electrogenic cells, first in cardiomyocytes [81], and more recently with neuron. Blaschke, Benno M., *et al.* [76] showed brain electrical activity mapping with a flexible EG-GFET *in vivo* in rats. It was demonstrated that graphene transistors offer additional advantages compared to existing state-of-the-art microelectrode-based recording technologies, such as intrinsic signal amplification and the possibility for down-scale and high-density integration.

GFETs for the detection of action potential from electrogenic cells were also reported. Extracellular signals from cardiomyocytes were detected with a high signal-to-noise ratio [81].

The detection of neurotransmitters is exceptionally challenging due to a low background concentration. Sensors for the detection of neurotransmitters need to have the option of being miniaturized. Besides the device’s size, flexibility is also essential for *in vivo* applications where good contact between the device and the tissue is required. Thus, this is where GFETs are highly promising.

Epinephrine neurotransmitter was detected by its oxidation on a FET gate electrode covered by graphene obtaining a LOD of 1 nM [80].

Dopamine sensing was performed with a reduced-graphene-oxide-based FET and obtained a LOD of 1nM with fast response [79].

Acetylcholine neurotransmitter was detected on polymer-modified CVD-grown graphene transistors functionalized with enzymes. Detection of this neurotransmitter was only possible at 0.5 μM concentration [77]. Enzymes as the functionalization method most likely caused this method's lack of sensitivity. An EG-GFET sensor based on electrochemical functionalization with enzymatic reaction also detected acetylcholine with a LOD of 2.3 μM with excellent selectivity [78].

GFETs used as biosensors are still in their early stages, however, provided the application of the inherent miniaturization potential of both FET devices and graphene channels, significant progress can be expected in the short future providing deeper results insights into biological processes. Hence, our interest in dopamine detection via GFETs.

Graphene's great qualities in biosensing make graphene-based devices more promising biomedical sensing contenders than silicon sensors. Silicon-based sensors' most significant drawback is their wafer production that relies on toxic products which reduces their intrinsic biomedical applications and silicon's reduced biocompatibility. Another major disadvantage of silicon-based devices is their temperature dependence which is very limited when used for *in vivo* monitoring [73].

4. FABRICATION OF GRAPHENE FIELD-EFFECT TRANSISTORS

Graphene biosensors dissemination is only possible with reproducible and scalable fabrication methods. Our transistors' fabrication protocol has been optimized and is reproducible, allowing the fabrication of almost a thousand chips for biosensing in only one wafer with over 80% yield. The miniaturization of these chips allied with a small acquisition platform and interface are of relevance for *in situ* measurements.

In this chapter we describe the fabrication of electrolyte-gated graphene field-effect transistors. Starting from graphene growth, wafer fabrication and everything related to our chips, from their layout and packaging to signal acquisition.

4.1. Graphene growth and transfer

Continuous monolayer graphene films were grown at INL's cleanroom facility by the 2D Materials and Devices group in an EasyTube ET3000 CVD system made by CVD Corp, USA.

The continuous single-layer graphene was synthesized on copper (Cu) substrates by chemical vapor deposition (CVD). The 10 x 10 mm Cu foils were initially chemically treated using a solution of FeCl_3 , HCl and deionized water for one minute in ultrasound to reduce rugosities and organic contaminations.

The copper (99.99 + % purity) foils were placed into a three-zone quartz tube furnace and the system was evacuated to approximately 2 mTorr and then filled with 250-sccm Argon (Ar, 99.999% purity) and 60-sccm Hydrogen (H, 99.999% purity) gas mixture. Once the growth temperature and pressure were reached, methane (0.5 sccm), the carbon precursor was introduced into the chamber. Graphene growth was carried out at 1040 °C with 6 Torr for twenty-five minutes on both sides of the copper foil.

For graphene transfer, a temporary poly(methyl methacrylate) (PMMA) substrate was used. PMMA (< 500 nm) was spin coated onto the top side of the graphene/Cu/graphene sample. Plasma ashing (PVA TePla GiGAbatch, 0.6 mTorr, O_2 :Ar 1:1, 250 W, 4 min) was performed to remove the graphene from the back side of the sample. Copper was then dissolved by dipping the PMMA/graphene/Cu into a 0.5 M FeCl_3 solution for two hours. And finally, PMMA/graphene was

cleaned in 2% HCl solution to remove metal precipitates and further washed in deionized water. Figure 11 is a picture of monolayer graphene.

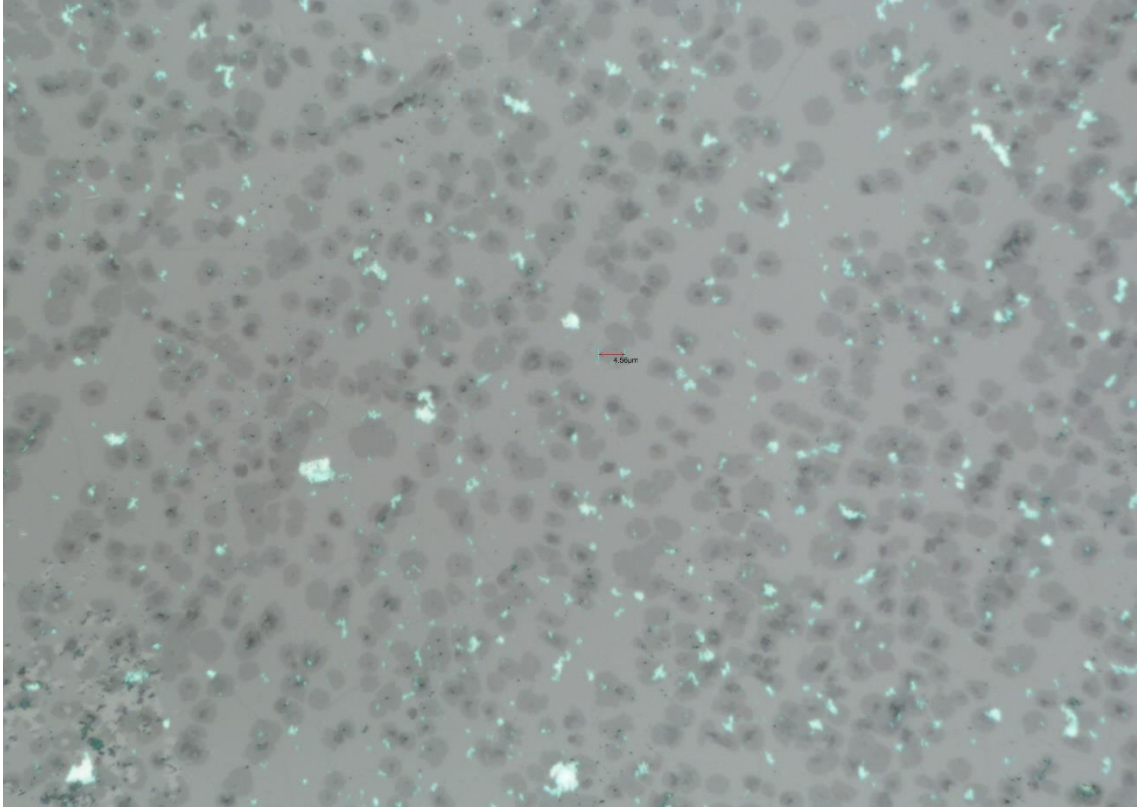


Figure 11. Monolayer graphene film on optical microscope with 50 x magnification.

4.2. Wafer fabrication

The fabrication sequence of the sensors' wafer used in this work, was performed at INL's cleanroom facility by the engineers of the 2D Materials and Devices group as previously reported [82]. The summarized sequence is as follows and is graphically depicted in figure 12. Each chip is composed of 20 sensors (EG-GFETs).

A 200 mm silicon (Si) wafer (p-type doped with boron, B) with 100 nm of thermal oxide was used as substrate. The wafer was sputter-coated with Chromium (Cr, 3 nm) as adhesion layer, and Gold (Au, 35 nm) as the conductive layer, and an alumina Al_2O_3 (20 nm) capping.

The source, drain and gate electrodes were patterned by optical lithography and ion milling. A sacrificial layer (TiWN, 5 nm; AlSiCu, 100 nm; TiWN, 15 nm) was sputtered and patterned by lift-off, for the material to be present where graphene is to be etched.

Previously grown graphene, as described above was transferred by wet transfer in cycles of FeCl_3 , HCl and deionized water. Graphene was then patterned using optical lithography and oxygen plasma etching, and the sacrificial layer was removed by wet etch. A protective layer (Ni, 10 nm; AlSiCu 30 nm; TiWN 10 nm) was sputtered and patterned by lift-off to work as a stopping layer for reactive ion etching.

The multi-stack passivation of SiO_2 and Si_3N_4 (total thickness of 250 nm) was then grown by plasma-enhanced CVD, patterned by optical lithography and etched, to cover the entire sensor area except only the graphene channel, and contact pads.

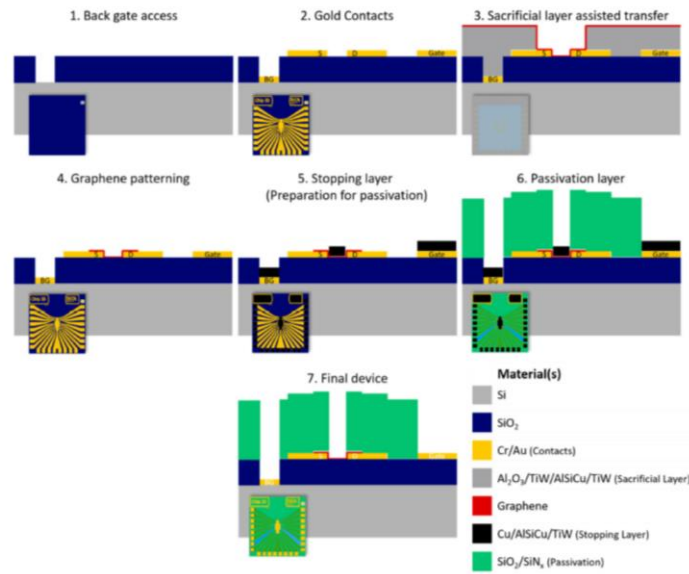


Figure 12. Graphical overview of the fabrication process of the EG-GFETs wafer [82].

The wafer was then diced and the sensors' photoresist was dissolved by acetone which was cleaned with propanol and deionized water. The chips were then glued onto a printed circuit board (PCB), wire-bonded with gold wires, and protected with silicone elastomer.

The materials directly exposed to the buffer and biological solutions in the experiments described in this dissertation are: Si_3N_4 , graphene and Dowsil 3140 RTV silicone elastomer.

Buried materials are p-doped silicon (bulk), silicon oxide, Au, Cr. Other thin-film materials deposited and removed during the fabrication have been verified to be absent in the final sensors by EDX analysis: Cu, Ni, AlSiCu, Al_2O_3 , TiWN; photoresist AZ1505 and PMMA have also been present at the exposed surface before removal by organic solvents. A photograph of the sensors' wafer is shown in figure 13.

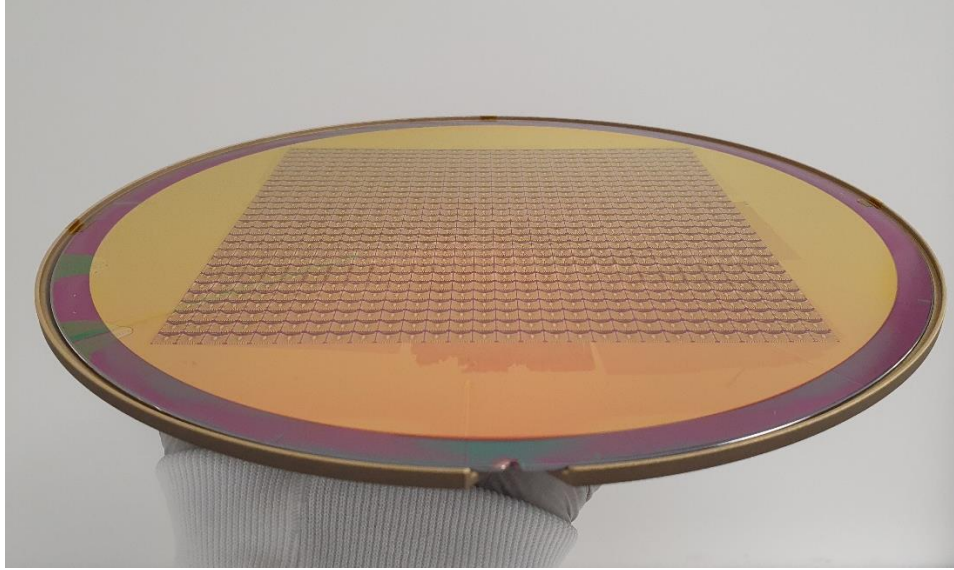


Figure 13. EG-GFETs' wafer.

4.3. Chips' layout

The wafer was diced into 784 equal-sized chips of 4.5 mm width and length. Each chip consists of one common in-plane gate electrode, and twenty graphene channels each with its drains connected to two different sources depending on its position. The layout of a chip can be found in figure 14.

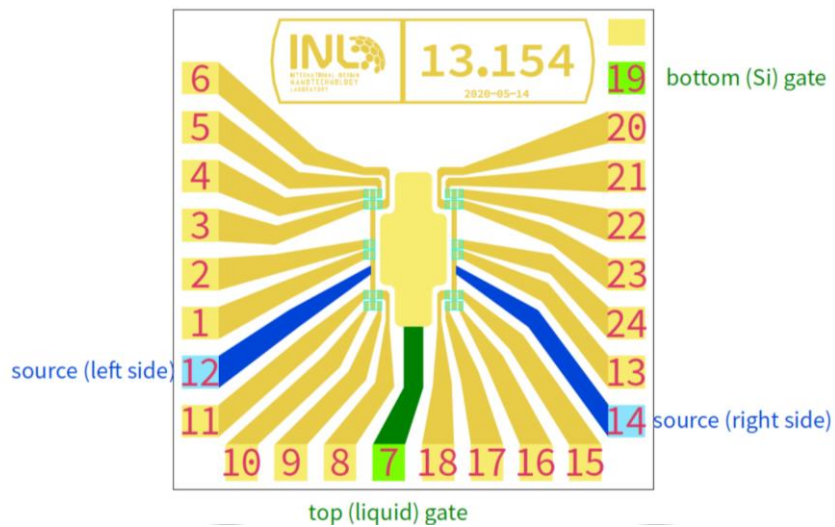


Figure 14. The layout of the EG-GFETs' chip. Drains (yellow pads), graphene channels (green rectangles), sources (blue pads), gates (yellow rectangle and green pads).

The photoresist that was coated as a final protective step of the wafer fabrication, was removed from the chips shortly before they were used to protect graphene for the longest possible

period. The photoresist was then dissolved in acetone, which was cleaned with a propanol solution and deionized water.

4.4. Chips' packaging

To allow interfacing the chips with an portable signal acquisition system, the chips were first glued to a PCB as showed on figure X, and then PCB's gold pads were connected to the chips' gold pads by wire bonding with the wire bonder HB16 (TPT HB) from INL's cleanroom facility.

Figure 15 shows the layout of the chip's PCB.

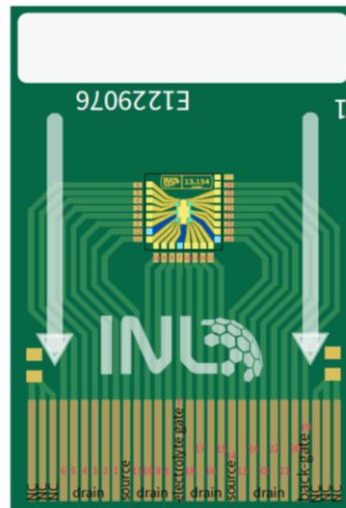


Figure 15. The layout of the chip's PCB.

The HB16 wire bonder is prepared for ball bond with 25 μm -thick gold wire. It accepts flat samples (for example, single-side PCB) up to 50 mm width, standard DIL chip carriers, and other standard packages. It is equipped with a heating stage to facilitate gold bonding.

The procedure for making the ball-stitch wire interconnection starts with the gold wire on the capillary tool being melted by an electrical flame-off (EFO) spark to form a gold ball at the end of the wire. The tool is lowered to the bond pad, and the gold ball is pressed against it. As the interface rises to the bonding temperature (from the heated holder), ultrasounds energy is applied forming the ball bond. The tool then is raised, leaving the ball welded to the tip's surface and forming a wire loop as it moves toward the second bond position, the chip's pad. The bond pad is positioned beneath the bonding tool, lowered to make the stitch bond. Afterward, the capillary tool

is raised, and a wire clamp pulls and breaks the wire-free and the bonder is ready to repeat the bonding cycle. Figure 16 shows the wire bonder machine.

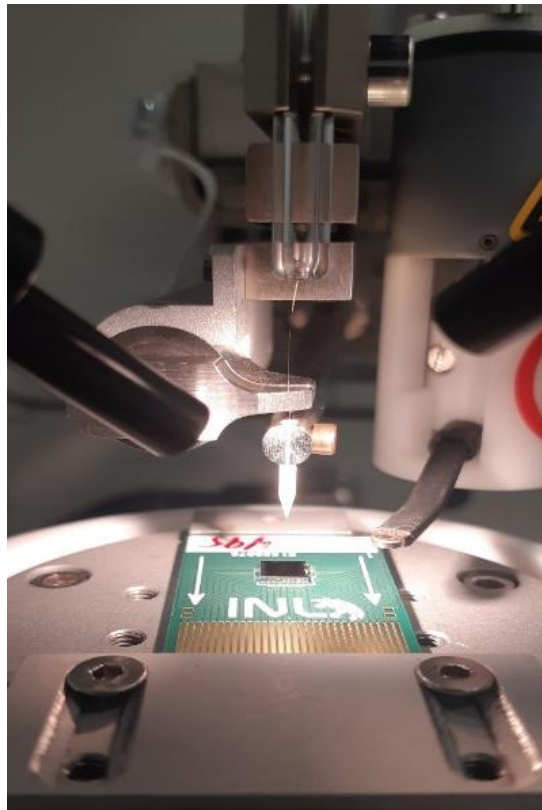


Figure 16. Wire bonder machine.

The gold ball bond recipe used for this wire bonding was the following. On the first bond, on the PCB's gold pad it was applied 450 mW ultrasounds (US) during 250 ms with a force of 600 mN. Whereas for the second bond, on the chip's gold pad, it was applied 300 US during 250 ms with a force of 400 mN.

Figure 17 shows a chip before and after silicone protection.

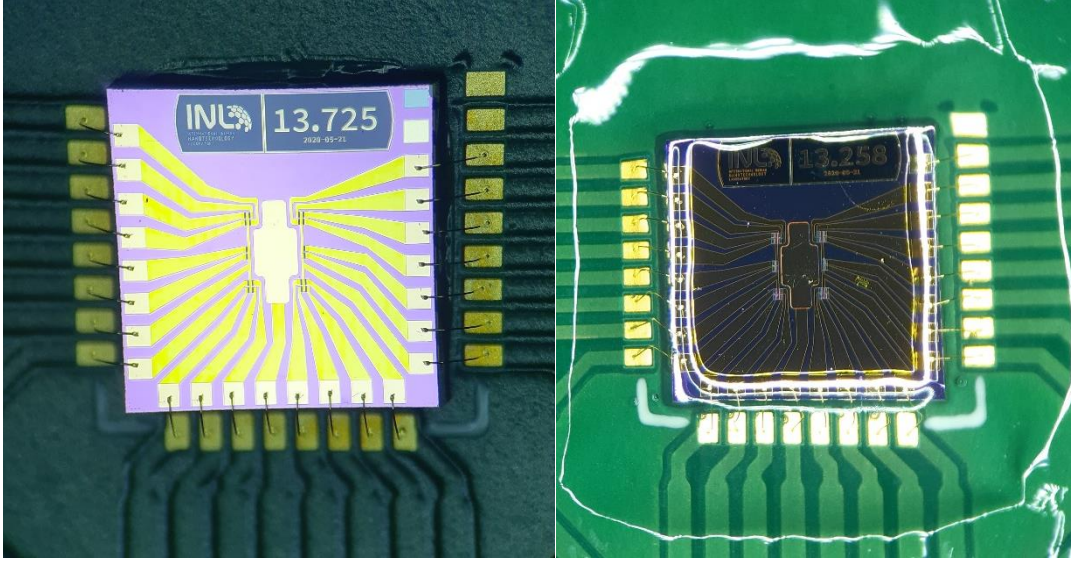


Figure 17. Chip with bonded gold wires (left) and a finished chip with gold wires and silicone protection (right).

If the transistors' pads are not fully protected by silicone, a current leak can occur if an electrolyte droplet is on top of the gate and pads. The current leak can happen between gate and source if the open (without silicone) pad is only the source. If the open pads are the source and drain, there is also another leak between gate and drains. Figure 18 shows an example of transistors' pads not fully covered by silicone.

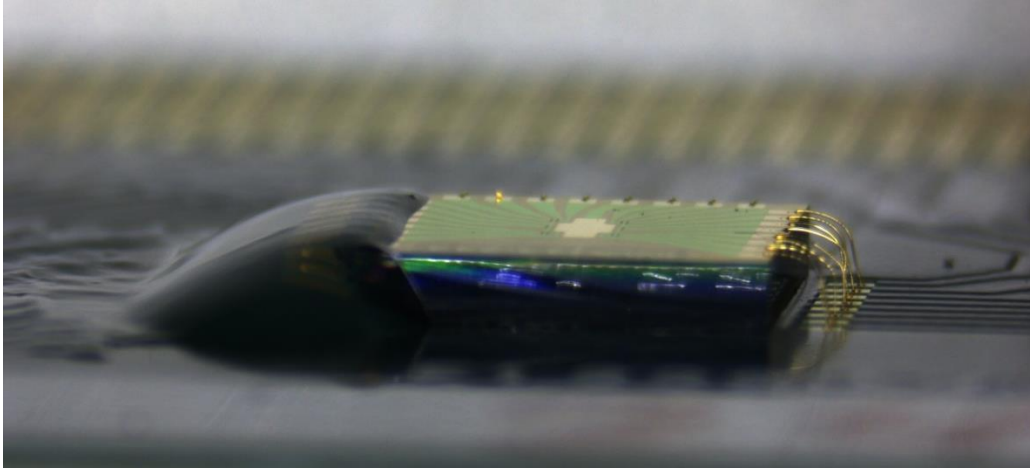


Figure 18. Chip with incomplete silicone coverage.

We theorized a model to obtain graphene's transfer curve in this scenario. To determine the current between drain and source, a PBS droplet was drop-casted on the chips' surface. To facilitate the calculations, the droplet was considered to have ohmic resistance value. When there

is only a current leak between gate and source, I_{DS} can be calculated by the sum of I_{DS} without PBS with V_{GATE}/R_{PBS} . The measured current will be higher than a no leak scenario. Such is confirmed for this situation in figure 19.

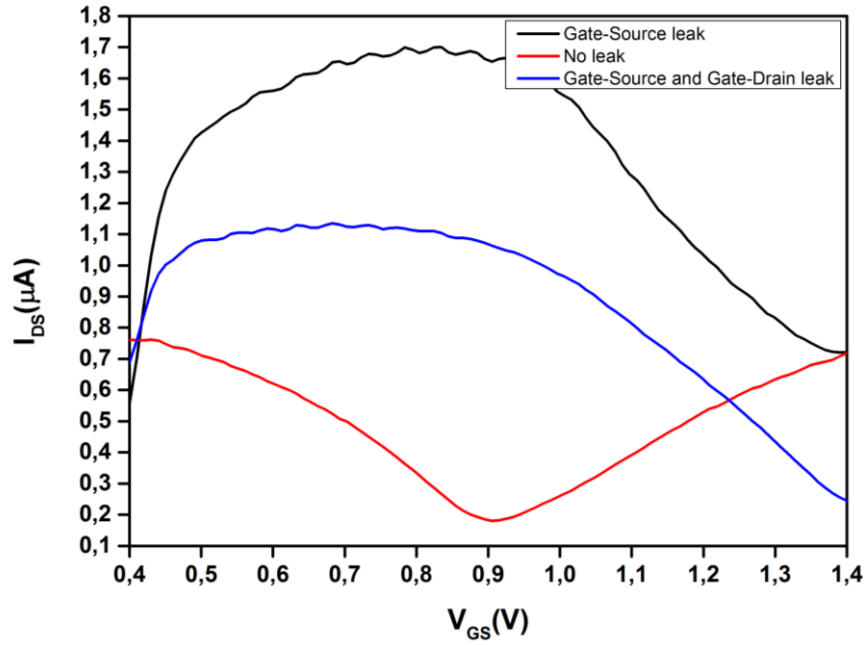


Figure 19. Graphic representation of I_{DS} dependence to different voltage leaks.

Figure 19 shows I_{DS} dependence to no leak, gate-source leak and gate-source and gate-drain leak scenarios. With this model we would have the sum of graphene's "V" shape with a linear ohmic behavior, a triangle shaped curve, which is not similar to the obtained one, however there is indeed a current increase.

This difference between the predicted curve and the obtained one can be explained by nonlinear effects of the electrolyte solutions and effects happening at the interfaces, especially for the situation where we have also a leak gate and drains.

4.5. Signal acquisition system

The signal acquisition platform that connects the chips to the computer interface, was designed, printed, and assembled at INL by the engineers from the Electronics group.

The PCB platform has a microcontroller that controls the Digital-to-Analog Converter (DAC) and generates a voltage. This voltage is applied for every device between the source and drain, and

the current is converted in voltage which is measured by the Analog-to-Digital Converter (ADC).

Figure 20 shows a photograph of the signal acquisition platform.



Figure 20. Signal acquisition platform.

The computer interface presents a real-time plot in Labview of the twenty sensors' transfer curves and an overtime plot of the Dirac point, such as the one shown on figure 21. For our measurements a V_s of 1 mV was set with a V_{GS} sweep from 0 V to 1 V. It was avoided to insert voltages higher than 1 V not to reach water electrolysis at 1.23 V, and the voltage starts from 0 V because for most transistors, having negative voltages induces more reading hysteresis.

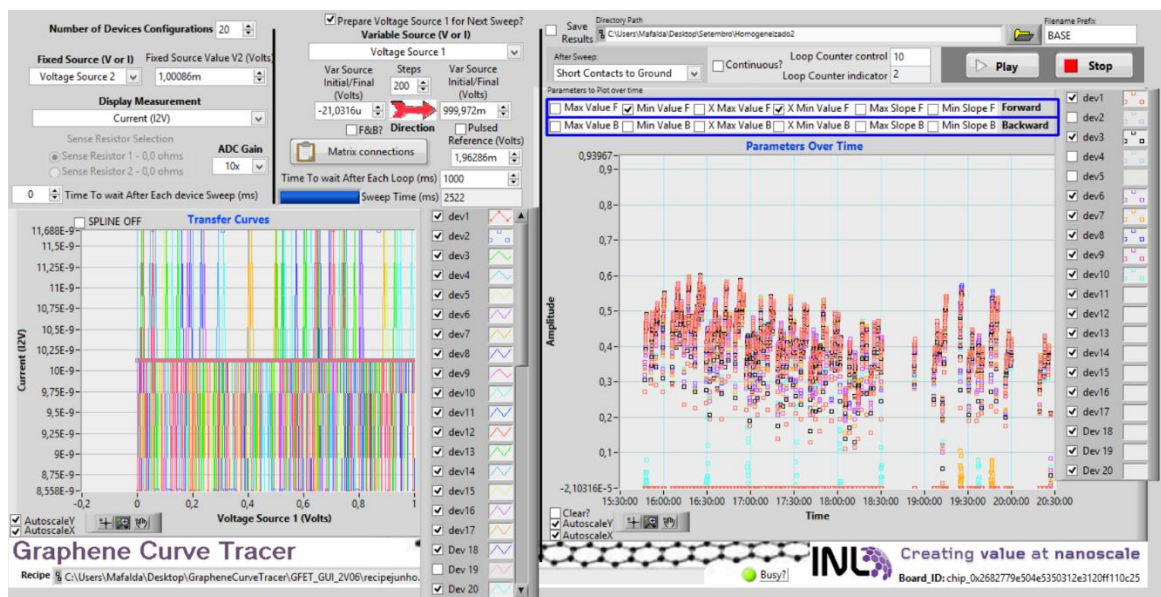


Figure 21. Signal acquisition interface.

The “prepare for voltage” mode, before each sensor sweep, puts each sensor at the first voltage value from the V_{gs} sweep range to stabilize the sensors for the desired starting voltage. The time to wait after each voltage sweep was 1000 ms, and the acquired data is an average of five ADC readings of gain ten.

Data acquisition for a transistor follows the matrix represented in figure 22. “Voltage 1” is applied to the gate (C7), and a voltage sweep is applied. The source, C12, is connected to “Voltage 2” where the V_s of 1 mV is fixed and the ADC reads the current between source and drain, C6, where current is converted to voltage.

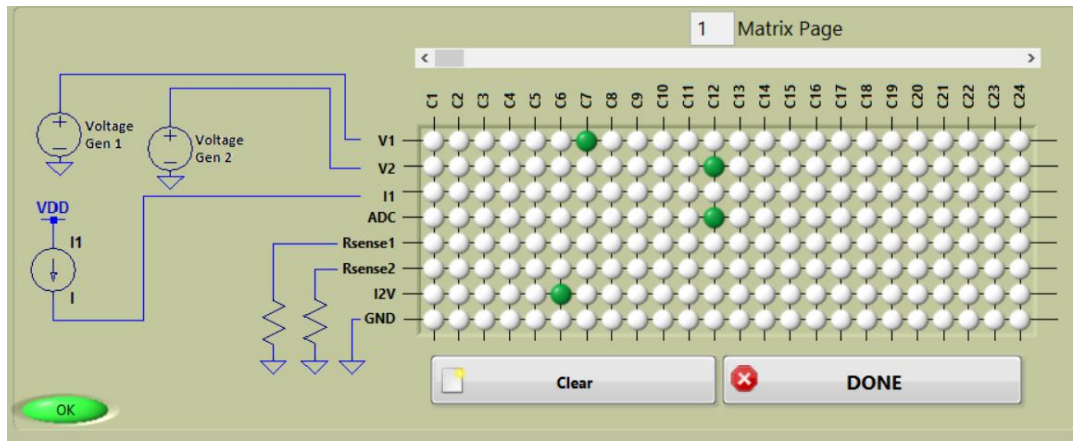


Figure 22. Data acquisition matrix.

The signal acquisition platform generates two files, one with the Dirac point of every measurement and the other with every point of the transfer curves.

Data acquisition was performed with only one loop to assess the electronics’ time to process the measurements. With only one loop of data acquisition, we should be able to determine the lowest acquisition time possible, this is of interest to us since in a near future, we would like to detect neurotransmitters as soon they are released from synapses. A synaptic event will occur below 1 ms.

The acquisition time presented on the generated file is a sum of the sweep time (time it takes to acquire data) and communication time (time it takes for the data to be sent to a excel file).

With the help of a digital oscilloscope, we input a known squared signal wave with set frequency and amplitude in the platform. With the oscilloscope probes precisely placed on our platform, we found out the time it takes to acquire two points by subtracting the area of the output

squared curve signal to the input one. The reason why we take two points is because of Nyquist's sampling theorem, which specifies that a sinusoidal function in time can be regenerated with no loss of information as long as it is sampled at a frequency greater than or equal to twice per cycle. We found out that our sampling time for 2000 points (measuring the highest possible number of points guarantees that there is no extra time breaking the points' acquisition loop) is 530 ms and the real acquisition time is 322 ms. In conclusion, the Nyquist frequency for our system is 300 μ s, but the time it takes to acquire one point is 0.15 ms, meaning that we can acquire data six times faster than the time a synapse takes to happen.

4.6. The chip signal

In an electrolyte-gated GFET (EG-GFET) the solid-state gate dielectric is replaced by an aqueous solution with a given ionic strength.

The gate voltage is transmitted through the electrolyte through electrical double layers. The electrical double layer (EDL) acts as a capacitor that charges when a gate voltage, is applied. A double layer is a structure that forms on a solid's surface when it is exposed to a fluid due to the different chemical potentials of the species in both phases. In polar solvents, like water, it starts with a single layer of solvent molecules with their dipoles aligned with the electric field in the space-charge region (the EDL). The first surface charge layer, either positive or negative depending on the balance between the chemical potentials, consists of counter ions firmly adsorbed onto the surface. The second charged layer is composed of ions attracted to the surface charge via the Coulomb force, electrically partially screening the first layer.

A gate voltage leads to a redistribution of the interfacial charge accumulated between the electrolyte and the graphene channel. The space-charge region, i.e., the EDL, acts as a capacitor's dielectric layer on top of graphene. The gate-voltage modulation of the space-charge layer changes its thickness and, therefore, its capacitance. Any change in the EDL capacitance is reflected in the transistor transfer characteristics.

Because the second layer of ions does not entirely screen the charge in the first layer, due to Brownian motion inside the liquid, the space-charge region extends towards the electrolyte's bulk, a distance is usually represented by the Debye screening length, λ_D . An electrolyte with a considerable Debye length is often preferable for liquid-gated FETs when used in bio-sensing. A larger λ_D provides a larger volume to accommodate molecules that bind to the molecular probes

on the graphene surface. The EG-GFET cannot detect reactions far outside the EDL because of the Debye screening length.

The following equation describes the Debye length (λ_D):

$$\lambda_D = \sqrt{\frac{\epsilon_r \epsilon_0 k_B T}{2 N_A e^2 I}}$$

Where ϵ_r represents the electrolyte's constant; ϵ_0 represents the permittivity of free space; k_B is Boltzmann constant; T is the temperature in Kelvin. N_A is the Avogadro constant; e is the elementary charge.

The smaller ionic strength gives rise to a larger λ_D , making the sensing probe-target interaction unscreened. Hence, as stated above, the sensing performance of EG-GFETs biosensors is susceptible to the ionic strength of electrolyte.

4.6.1. Transistor transfer curve physics

Graphene's transfer curve has a "V" shape where each branch represents the excursion of the Fermi level inside a different electronic band. According to the popular Drude theory, it is common to associate transport in the valence band (the left branch of the transfer curve) to hole carriers and the conduction band to electrons (the right branch of the transfer curve).

Our as-fabricated graphene FET are p-doped, a consequence of lithographic processes, and consequently, the charge neutrality point is found at a positive gate-source voltage. For most chips, the transistors first measurement (bare graphene without any functionalization step) displayed a Dirac point around 0.6 V, shown in figure 23.

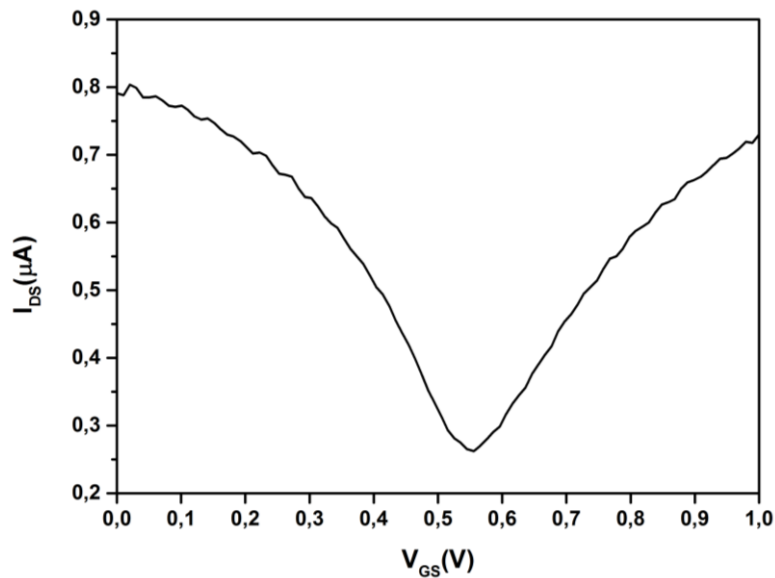


Figure 23. Graphene transfer curve without functionalization, I_{DS} as function of V_{GS} .

4.6.2. Ionic buffer solutions dependence

Since the ionic strength affects the Debye length and the electrical response of the electrolyte-gated biosensors, we studied the effect of using phosphate buffered saline (PBS) with different concentrations, i.e., different ionic strengths in the response of the transistor. It is reported in the technical literature that PBS 1x has an ionic strength of 162.7 mM and a λ_D of 0.76 nm; PBS 0.1x has an ionic strength of 16.27 mM and a λ_D of 2.41 nm [83].

Figure 24 shows the graphene's transfer curve for two PBS concentrations.

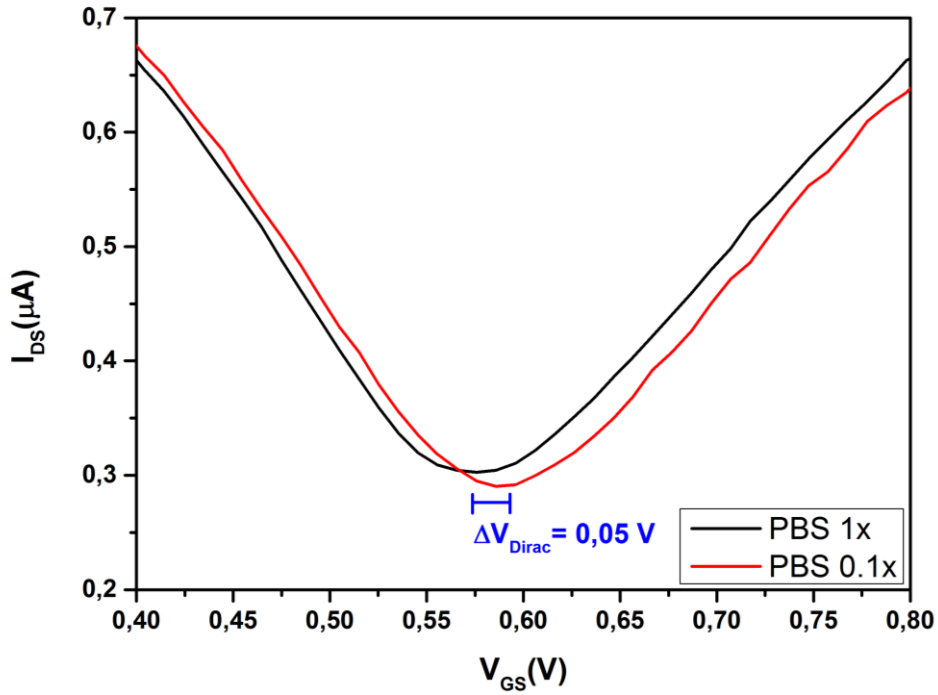


Figure 24. Graphene transfer curve, I_{DS} as function of V_{GS} for different electrolyte strengths.

PBS 0.1x produces a positive Dirac point shift of fifty millivolts relative to PBS 1x. Since the ionic strength of the solution is lower, the Debye length is higher and the EDL capacitance is smaller. Consequently, higher voltage is required to accumulate the same number of charges in the channel, which means that the same current level is obtained at a higher V_{GS} .

4.6.3. Overtime buffer measurements

We studied the effect that water evaporation would have on our measurements. To that goal, a volume of 20 μL of phosphate-buffered saline 1x solution was drop cast on a chip and data were acquired overtime as the water evaporated from the droplet. The gate-source voltage was continuously swept from -0.2 V to 0.8 V.

Within one hour of evaporation and continuous data acquisition, some effects become noticeable, as the droplet saturates and the salt precipitation is potentiated, increasing the ionic strength of the medium and giving rise to a transfer curve drift to lower V_{GS} , as shown in Figure 25, for the reasons discussed in the previous paragraph.

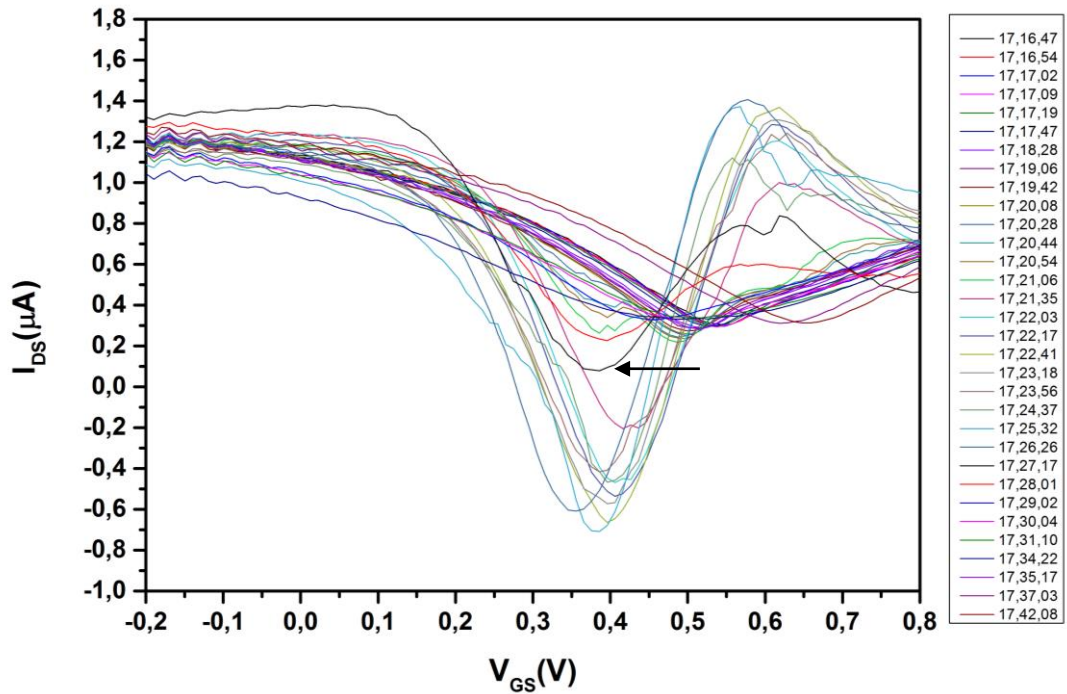


Figure 25. Graphene's transfer curve with I_{DS} as a function of V_{GS} overtime. Data were acquired continuously over a time interval of half an hour. Black arrow indicates the direction of the data acquisition.

When the water droplet becomes flattened and salt deposition occurs substantially on the graphene channels, the transfer curve loses its “V” shape, and negative source-drain currents are observed. New peaks start to built-up at higher V_{GS} becoming the new current maxima.

When the droplet completely dries, the curves become since there is no transistor action because the gate dielectric is lacking. The transistors' current flow is instantaneously restored as soon as the PBS droplet is renewed. However, because the chip's surface is not cleaned before a new electrolyte volume is dropped, the solution's ionic strength is higher than the initial one, and therefore new set of curves is centered at a different (lower) gate voltage.

The explanation for the negative values of I_{SD} and the overall new shape of the transistor transfer curve whence the water droplet is about to evaporate fully cannot be found in the acquired data. An $I_{DS} < 0$ means that there is a current source in addition to the FET source contact.

This additional source probably stems from redox reactions affecting the highly concentrated salts in the almost entirely evaporated water droplet.

The V_{Dirac} stability was tested for many V_{GS} cycles in a constant voltage range, for $-0.2 \text{ V} < V_{\text{GS}} < 0.8 \text{ V}$, $-0.1 \text{ V} < V_{\text{GS}} < 0.9 \text{ V}$, and $0 \text{ V} < V_{\text{GS}} < 1 \text{ V}$ in PBS 1x. The transfer curves were continuously acquired for 10 minutes, without allowing the liquid-gate to evaporate, for each V_{GS} interval (see figure 26). V_{Dirac} is stable in time for the first two intervals, after a small initial drift. For the last interval, V_{Dirac} drifts in time, from $\sim 0.70 \text{ V}$ to $\sim 0.82 \text{ V}$. Comparison between the time series shows that V_{Dirac} average values change from series to series.

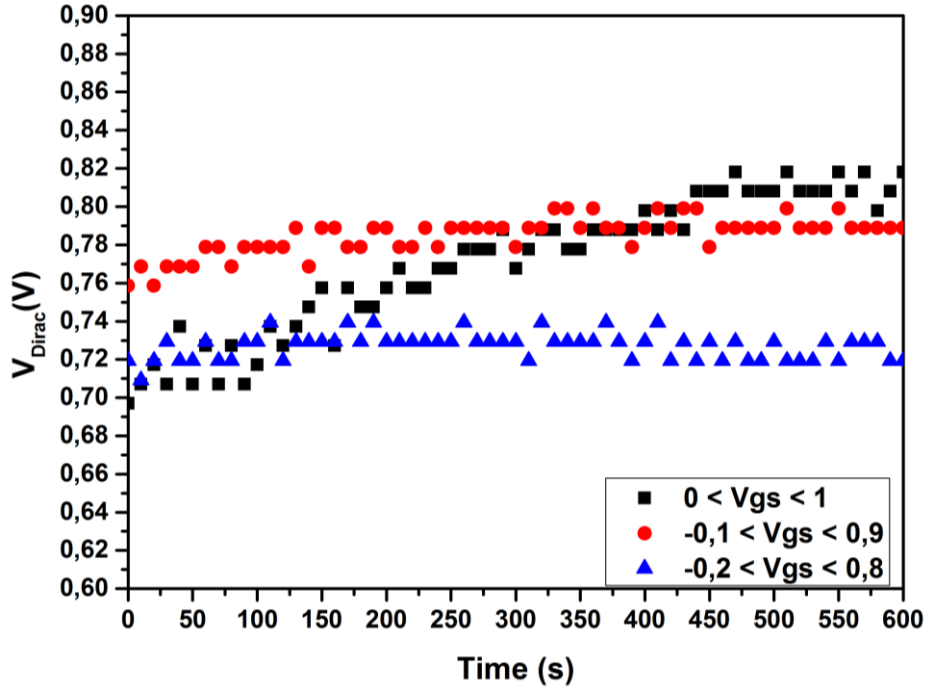


Figure 26. Dirac point time dependence as function of V_{GS} . V_{GS} sweep from -0.2 V to -0.8 V (blue), -0.1 V to -0.9 V (red) and 0 V to 1 V (black).

We see no reason for the inter-time series Dirac point instability, but we suspect it is associated with the acquisition platform.

In order to assess our sensor's measurement error, we acquired transfer curves for six transistors of the same chip over time. By doing overtime measurements in a very controlled medium, minimizing external factors' interference such as temperature, droplet volume, pH alteration, and salt precipitation, we can determine the transistors' measurement fluctuations and consider it our sensor's error.

This chip had a $20 \mu\text{L}$ droplet of PBS 1x as the gate electrolyte, and the volume was kept over time thanks to a controlled humidity chamber. Four sets of six V_{Dirac} measurements were acquired for set times, where each point is an average of 5 acquisitions.

The average difference from the first measurement to the last one, for the six sensors averages, was 4 mV, meaning that this should be considered our measurements' error and should be taken into account when analyzing Dirac point shifts of similar magnitude. Such data can be found on figure 27.

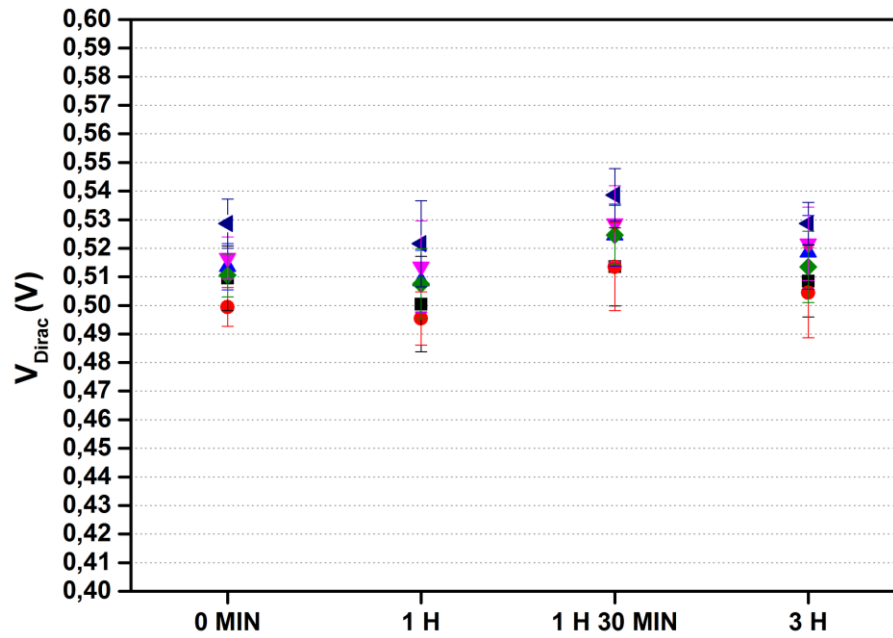


Figure 27. Dirac point time dependence for the same PBS 1x droplet. Each data point is a measurement from one transistor.

5. SURFACE FUNCTIONALIZATION AND PASSIVATION METHOD

To achieve a biosensing system based on EG-GFETs, first, we need to functionalize the active transducing area of the biosensor, the graphene FET channel, with a selective biorecognition element. A dopamine-specific DNA aptamer was chosen for its ability to interact with the graphene channel within the Debye length and for high selectivity. As graphene is chemically stable and covalent modification of the material alters its electrical properties, a non-covalent method was used to modify graphene. This chapter describes the functionalization and passivation processes of our sensors and spectroscopy techniques to prove the successfulness of our method.

5.1. Functionalization process

The full functionalization process can be found on figure 28.

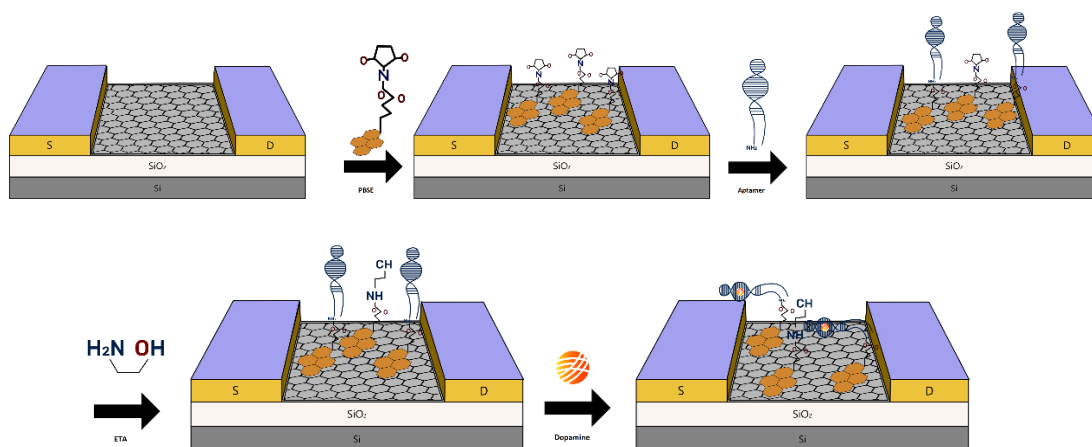


Figure 28. A graphical depiction of the full functionalization process. DDT passivation (first), PBSE probing (second), aptamer binding (third), ethanolamine blocking (fourth) and dopamine binding to the aptamer (fifth).

Initially, to prevent non-specific interaction of biological sample and restrict functionalization to the graphene channel, the Au receded gate is passivated with 2 mM of 1-Dodecanethiol (DDT), a gold blocking agent, in ethanol for 4 hours. Between every functionalization step the chip's surface is cleaned with deionized water and blown dry with N₂ flow.

To facilitate the DNA immobilization to the graphene, a pyrene-derivative, 1-Pyrenebutanoic acid N-hydroxy-succinimide ester (PBSE) diluted in N, N-Dimethylformamide (DMF) was used as a bifunctional linker to create an interface between graphene and the biosensing probe, 20 μ L of 10

mM of PBSE was incubated onto the graphene channel for 2 hours. The pyrene group of PBSE establishes a very stable non-covalent interaction with graphene (π - π interaction) due to its structural similarity with graphene and remains with a free ester group that will allow the N-Hydroxysuccinimide reaction (NHS) (reaction where ester-activated crosslinkers react with primary amines in physiologic to slightly alkaline conditions, pH 7.2 to 9, to yield stable amide bonds) with the amine group of the aptamer, the sensing probe. This way, the aptamer will be firmly bond with the PBSE linker without altering the electronic properties of graphene.

Two different dopamine-specific DNA aptamer sequences were studied. After probe bonding to PBSE the following aptamer sequence produced the largest Dirac point shift in regard to the PBSE's Dirac point. The 44-mer sequence of DNA aptamer with 5' C6-aminolink modification (5' - CGACGCCAGTTTGAAGGTCGTCGCAGGTGTGGAGTGACGTCG -3') [15] was synthesized by STABvida. Despite being 57-mer long, the other DNA sequence [15] did not produce a big Dirac point shift as the shorter one for the same electrolyte solution. Having more nucleobases one would expect to see a larger Dirac point shift, since we have more charges the screening effect on the graphene would also be more intense and therefore bigger Dirac point shift. However, that was not the case, a possible reason is that for the same Debye length, only the charges located within such distance, contribute for the screening effect on graphene, and so, the rest of sequence's length located above the Debye length does not contribute.

The dopamine-specific aptamer was then prepared by diluting 20 μ M solution of aptamer in MilliQ water and by heating the final solution to 95°C for 10 minutes. This heating procedure removes misfolds in aptamer structures and maximizes correctly folded secondary structures. The aptamer was only dropped on the chip after cooling down to room temperature. For incubation, 20 μ L were added on top of the chip and kept for 16 hours in a humid chamber in the dark. Different aptamer concentrations and incubation times were studied, and this set of parameters (20 μ M with 16 H incubation) have given the best PBSE-aptamer binding results since they displayed the biggest Dirac point shift in regard to previous functionalization step.

After the aptamer immobilization, some free ester groups from PBSE can remain active on the surface, thus to minimize potential non-specific binding, 20 μ L of ethanolamine (ETA) were dropped on the chips and incubated for 30 minutes. ETA is a small molecule with an amine group in one extremity that will react with PBSE's free ester groups, and a hydroxyl group that blocks the reaction of the linker with dopamine and others. The non-ligand PBSE blocking step is the last stage from the surface functionalization process.

Every stage of the functionalization process was verified with electrical measurements by measuring the Dirac point shift in respect to the previous step. Additionally, to confirm PBSE-aptamer binding, X-Ray Photoelectron Spectroscopy (XPS) and Confocal Raman Spectroscopy experiments were performed on graphene substrates with this functionalization method.

Figure 29 shows graphene's Dirac point and full transfer curve for every functionalization step.

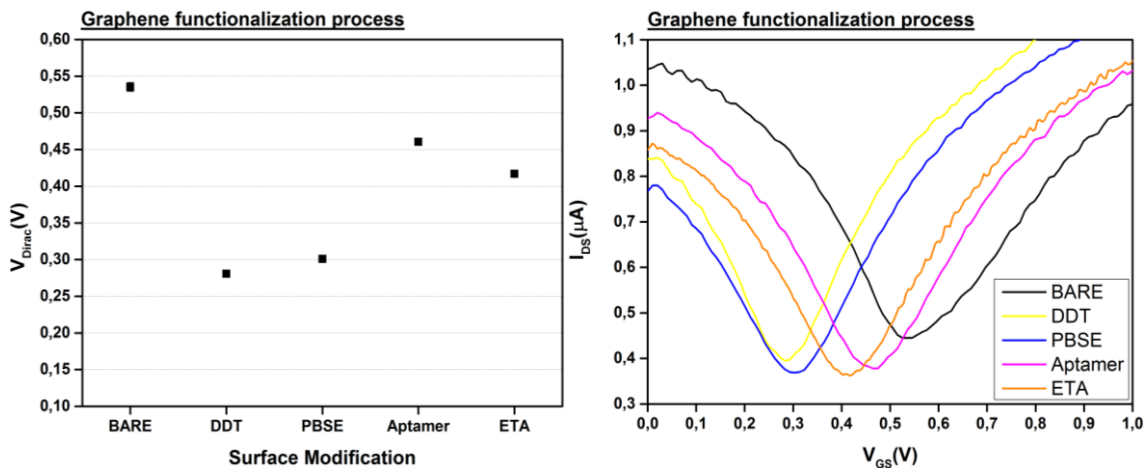


Figure 29. Dirac point as function of surface modification stages (left) and characteristic transfer curves after each stage of the EG-GFET channel functionalization.

Every functionalization step electrical measurement was made with a 20 μL PBS 1x droplet on the chip.

The graphene transfer curve is symmetric around a point of minimum I_{DS} , occurring at a gate voltage V_{Dirac} , where the channel conductivity is at its lowest, corresponding to an electron chemical potential (Fermi level). The steep branches in the curve, to the left and right of V_{Dirac} , correspond to transport by holes and electrons, respectively.

On the first measurement, where graphene hasn't been functionalized (BARE), the position of V_{Dirac} is located on the positive values of gate voltage (540 mV), this shows that our graphene is p-doped. This non-intentional doping likely originates from residues of process chemicals and is common in CVD-graphene processed by wet transfer.

The gold passivation step, DDT, moves the Dirac point to 280 mV, this shift of approximately -300 mV results from a change of composition of the gate surface, with a formation of a self-assembled monolayer that covers the Au contact.

This process is accompanied by dipole formation at the interface, induced by charge transfer during chemisorption of sulphur on Au on the combined dipole moments of the alkane chains. For

common alkanethiols, like DDT, this dipole moment is oriented from the surface into the solution [86].

Consequently, there is a positively charged layer inside the solution, bound to the surface of the Au, held at V_{GS} by the power supply, which increases the electric field resulting from the applied gate voltage ($V_{GS} > 0$). Therefore, gating is enhanced, and the transfer curve is shifted toward lower values of V_{GS} .

The functionalization of carbon surfaces with 1-Pyrenebutanoic acid N-hydroxy-succinimide ester (PBSE) leads to small shifts in chemical potential [62]. We observed a shift of approximately +20 mV in respect to the previous Dirac point (DDT). The sign of the transfer curve shift toward more positive V_{GS} , indicates that there is additional p-doping of the graphene upon π - π stacking of the linker. Through XPS experiments, the graphene surface coverage with the PBSE linker proved to be around 60% of the total graphene area (data not shown).

When the aptamer is immobilized onto the PBSE, there is a +160 mV shift of the Dirac point. Aptamer being DNA is a negatively charged molecule at neutral pH. The bonds created between oxygen and phosphorus atoms are negative, which makes the phosphate backbone of DNA negative. Hence, the screening effect on graphene gives a positive V_{DIRAC} shift.

The last functionalization stage, PBSE blocking by ethanolamine, mitigates the effect of the linker that did not bind to the aptamer. Therefore, we see a slight move of the Dirac point to lower voltages, a -40 mV shift.

5.2. Assessment of the functionalization process

5.2.1. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) was performed to verify aptamer binding to the PBSE linker. Experiments were carried out on the XPS system ESCALAB 250X by Thermo Scientific at INL.

A thermal p-doped silicon oxide wafer was coated with AZ1505 photoresist and then cut in 1 mm² area sample substrates. All samples had monolayer graphene transferred on to them with the same transfer process that was done on the sensors' wafer.

The first sample was incubated with PBSE linker for two hours and the second sample was incubated with the dopamine-specific aptamer for sixteen hours, both being light protected. These experiments were repeated at least three times per sample type.

For the PBSE molecule, we were expecting to see an increase in oxygen and carbon when compared to the substrate only, and the emergence of nitrogen. The acquired data showed the following atomic percentages: O 1s 25.77%, C 1s 70.79%, N 1s 3.14% and P 2p 0.3%. The high intensity for carbon peak is explained by the graphene presence on the substrate, whereas oxygen and nitrogen come from the linker's ester group. PBSE also contributes with carbon but only marginally when compared with the substrate. The atomic percentage found for phosphorus was considered to be from the wafer p-type.

Aptamer being DNA, we were expecting to be able to detect oxygen, carbon, and phosphorus. The atomic percentages for the aptamer sample were the following: O 1s 31.25%, C 1s 73.41%, N 1s 3.07% and P 2p 1.25%.

		Height CPS	FWHM eV	Area (P) CPS.eV	Atomic %
PBSE	O 1s	138359,14	3,08	470188,9	25,77
	C 1s	137259,15	2,92	509376,12	70,79
	N 1s	7954,53	3,3	35815,32	3,14
	P 2p	489,15	2,5	3105,26	0,3
Aptamer	O 1s	155742,38	3,02	541535,84	31,25
	C 1s	155495,53	2,97	557787,48	73,41
	N 1s	5938,77	4,53	36951,1	3,07
	P 2p	1655,57	8,32	12324,35	1,25

Figure 30. XPS peak parameters for O 1s, C1s, N1s, P2p for PBSE and Aptamer on silicon substrate.

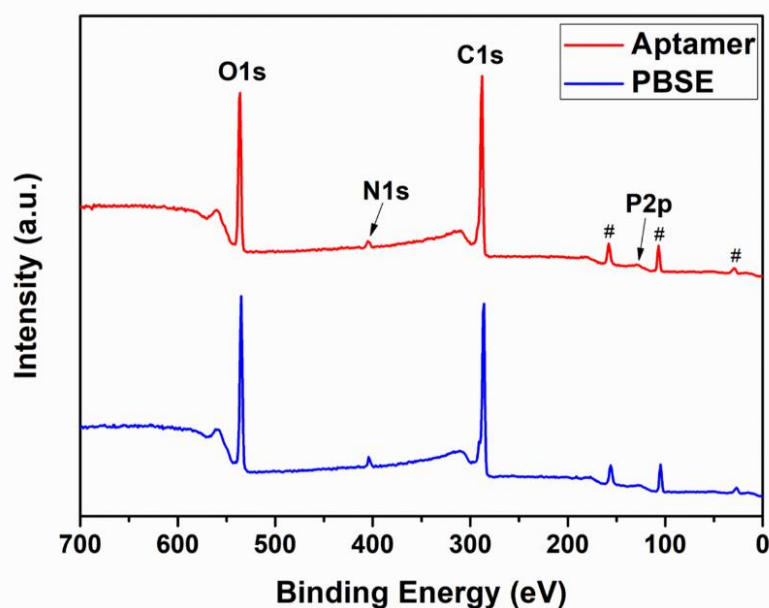


Figure 31. XPS survey graph of a PBSE sample (blue) and Aptamer sample (red).

From the samples' surveys and parameters' table we can see an increase on every peak of interest. The atomic element represented by # is Silicon which was present on the substrate and was not altered from sample to sample.

Carbon 1s spectrum analysis

There are two ways for DNA to bind to PBSE, either to the carbonyl group (C=O) or to the carboxylate (C-O) part of the linker.

In the first case, when the amino termination of DNA binds with C=O, the C=O peak decreases in the C 1s spectrum. On the second case, the amino group binds with C-O and this oxygen leaves and binds to the aromatic nitrogen group. Due to this reason, we can observe that the (C=O)-O peak decreases.

New contributions are expected to be observed at a binding energy of around 286.5 eV [84], which can be attributed to the carbon atom attached to oxygen forming C-O-C (epoxy carbon) and the C-OH bonds in the sugar unit from DNA. The binding energy of photoelectrons ejected from these species corresponds to a value of electron binding energy from a C-N bond.

The C-O bond now becomes C-N bond due to the two new contributions C-O-C and C-OH.

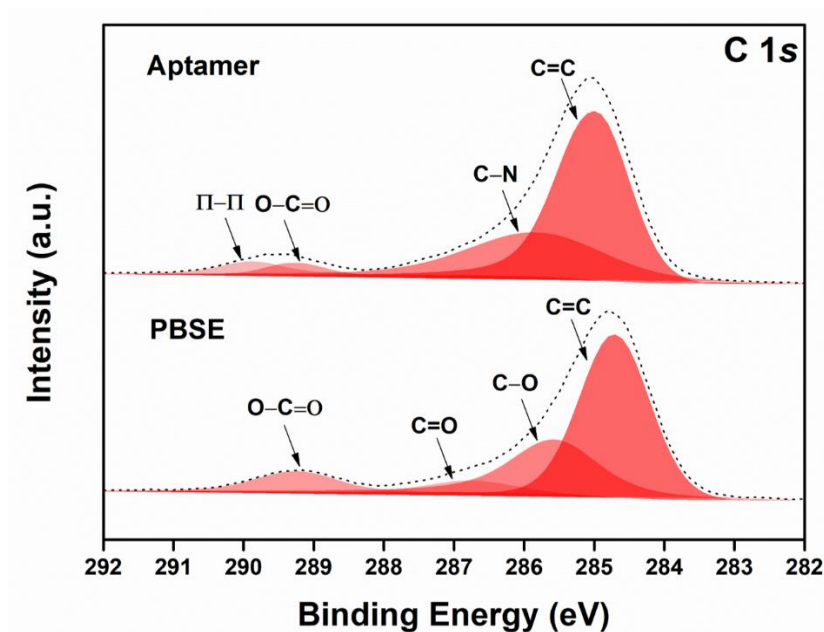


Figure 32. Carbon 1s spectrum.

Carbon 1s had an increase, in regard to its measurement from PBSE sample, in its atomic percentage from 70.79% to 73.41%, with the observed peaks being higher, and with an increase in area from 509376,12 CPS.eV to 557787,48 CPS.eV.

Nitrogen 1s spectrum analysis

From the PBSE linker, it is expected to find N-(C=O)-O- at 401.5 eV and an aromatic ring peak around 399.5 eV [85]. The DNA binds to the linker via amino group on the C-O strand of the linker and, thus, we observed a decrease of the N-(C=O)-O- bond and increment in the aromatic ring peak. This observation may be attributed to the addition of the amine termination from the DNA strand, which is expected to be around 399.4 eV.

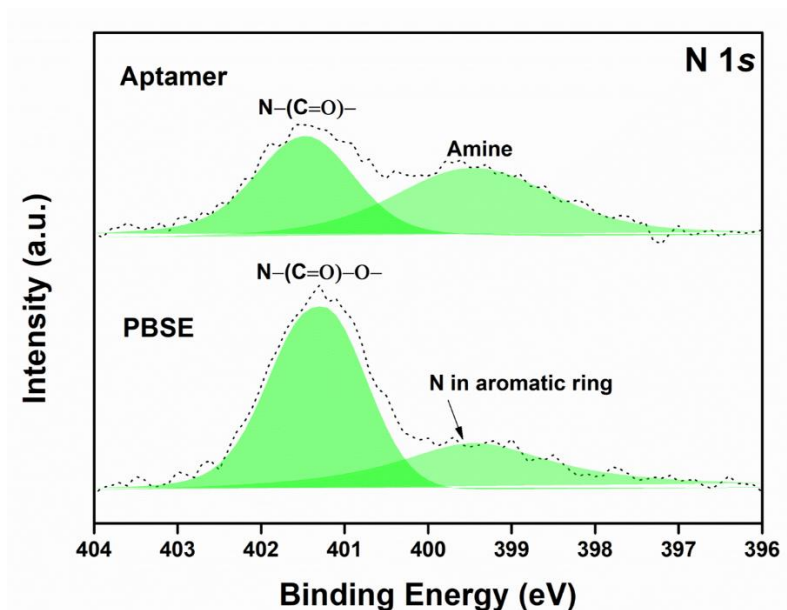


Figure 33. Nitrogen 1s spectrum.

Nitrogen 1s had a decrease of its overall peaks' height, in regard to PBSE's sample peaks, from 7954,53 CPS to 5938,77 CPS but the total area increased from 35815,32 CPS.eV to 36951,1 to CPS.eV. The atomic percentage remained unaltered.

Oxygen 1s spectrum analysis

Oxygen spectrum shows two peaks, the most intense being the C=O bond peak at 533 eV from PBSE's aromatic ring, and the least intense being the H-C-O bond from PBSE's strand that will link directly to the amino group from DNA, with binding energy around 535 eV.

The H-C-O bond that was present on the PBSE sample disappears as DNA's amino group binds to the linker, and additional C=O bonds of the DNA strands are observed. The C=O peak had an area of 470188,9 CPS.eV and height of 138359,14 CPS in the PBSE sample. With linked DNA, this peak increased to an area of 541535,84 CPS.eV and 155742,38 CPS height.

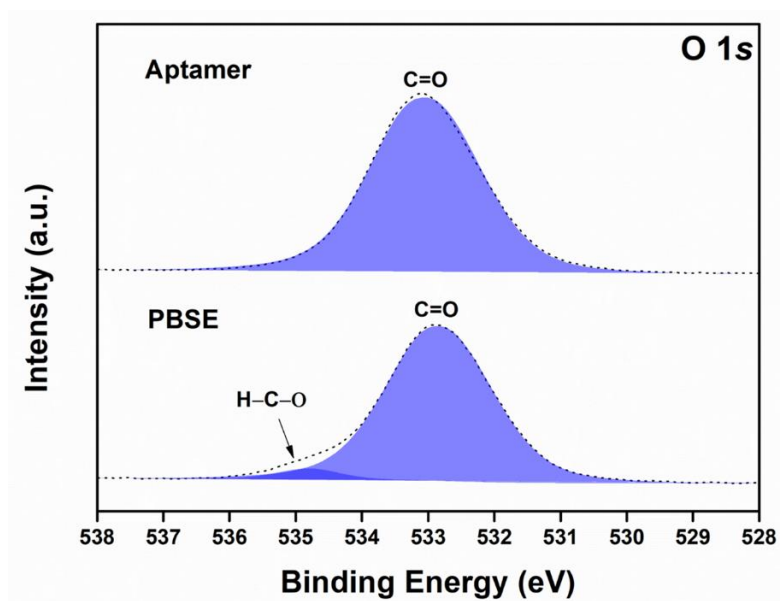


Figure 34. Oxygen 1s spectrum.

Phosphorus 2p spectrum analysis

DNA contributes with PO_4^{3-} , phosphate, from its phosphate-deoxyribose backbone, with characteristic binding energy at around 134 eV [84].

An additional intense peak was found at 129 eV which may be attributed to the phosphorus atom.

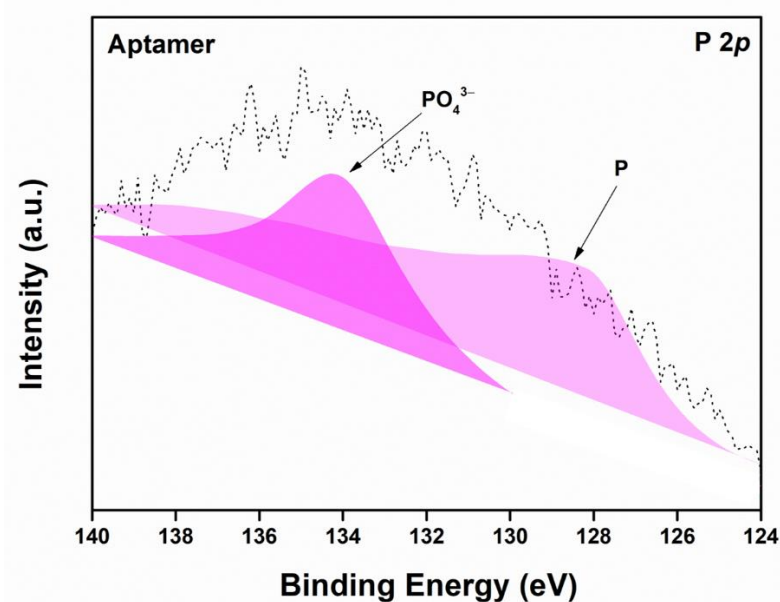


Figure 35. Phosphorus 2p spectrum.

Phosphorus 2p peak's height increased, in regard to PBSE's sample peak, from 489,15 CPS to 1655,57 CPS, area increased from 3105,26 CPS.eV to 12324,35 CPS.eV, and the atomic percentage increased from 0.3% to 1.25%.

5.2.2. Raman spectroscopy

Confocal Raman spectroscopy (Alpha 300R, WITec) experiments were carried out at INL. Raman data analysis and imaging were done with Project FOUR+ software (WITec).

Raman spectroscopy has historically been used to probe structural and electronic characteristics of graphite materials, providing useful information on the defects (D-band), in-plane vibration of sp² carbon atoms (G-band), as well as the stacking orders (2D-band) [87].

The chip's monolayer graphene channel and dopamine hydrochloride in powder form and diluted form spectrums were analyzed.

In the figure below, the red spectrum represents monolayer graphene, where the 2D mode at 2700 cm⁻¹ is bigger than the G mode at 1580 cm⁻¹ indicating that our channel's graphene is indeed monolayer [88]. Blue spectrum is dopamine's Raman signature on glass substrate and pink, green and cyan are all dopamine diluted on PBS 1x on a graphene substrate. The wide peak at around 3400 cm⁻¹ is characterized by water and the pink spectrum was taken a few minutes later after green and cyan, the peak has decreased which reflects the droplet evaporation.

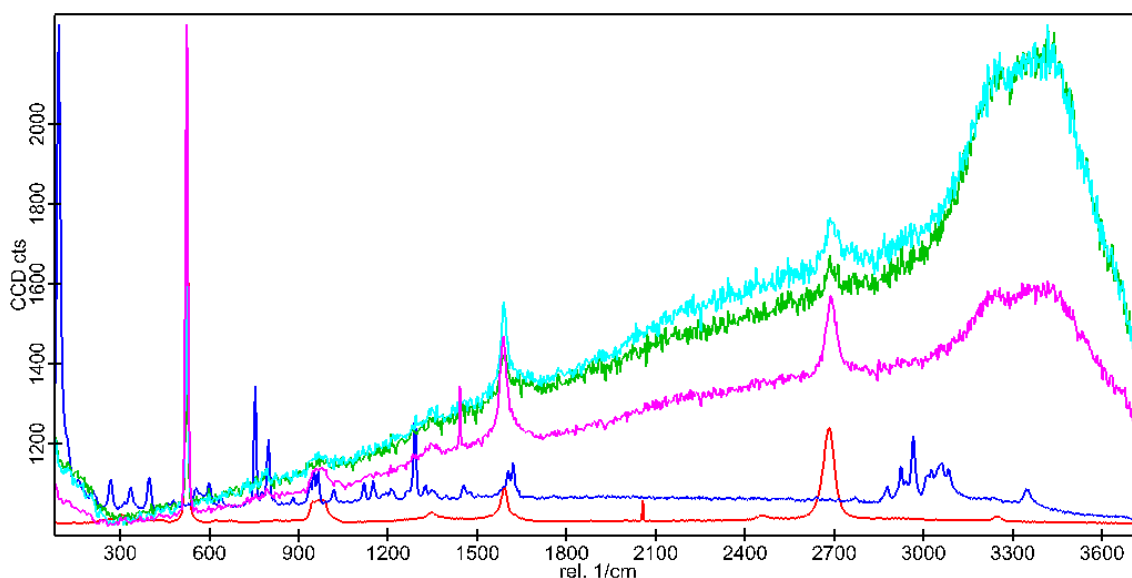


Figure 36. Monolayer graphene, dopamine in powder and diluted dopamine spectrums. Graphene (red), dopamine powder form (blue) and dopamine diluted in PBS (cyan, green and pink).

Such Raman measurements were able to confirm the presence of monolayer graphene in our transistors' channels and to analyze dopamine's Raman spectrum. However, the Raman acquisition system lacked the sensitivity to detect dopamine's spectrum after washing and drying the chips after having dopamine incubated on them. Such experiment could have better results when carried out with surface-enhanced Raman spectroscopy, because it is a technique that offers orders of magnitude increases in Raman intensity.

Dirac point voltage variation and x-ray photoelectron spectroscopy successfully proved aptamer binding to PBSE, and thus, our functionalization method.

6. DOPAMINE DETECTION *IN VITRO*

Initial *in vitro* experiments with optimized buffers are fundamental to study biosensors' responses in a controlled environment, as well as to establish calibration curves. Thus, in this chapter, dopamine detection *in vitro* is analyzed for phosphate buffer saline solution and artificial cerebrospinal fluid electrolyte solutions. Dopamine effects on our sensor's surface with different passivation methods are presented, as well as our sensors response to dopamine's analogues and biological interfering agents'.

The data described in this chapter was presented in "NanoPt2021" conference (see abstract on Supplement 1).

6.1. Dopamine detection in phosphate buffer saline (PBS)

Dopamine hydrochloride [Sigma-Aldrich] was weighed and diluted in phosphate buffer saline 1x into solutions of different dopamine concentration, ranging from 0.1 zeptomolar (10^{-22}) to 0.1 millimolar (10^{-4}). PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM) at 7.4 pH is commonly used as an electrolytic buffer solution in the experimental assessment of biosensors because is the pH found in biological fluids [89].

Measurements were performed with 20 μL droplets of each concentration pipetted on top of the transistors.

Figure 37 shows dopamine detection as function of ΔV_{DIRAC} . Plotted data is an average for 416 transistors that yielded reliable data from around 60 chips. Every dopamine concentration is an average value of at least 80 transistors.

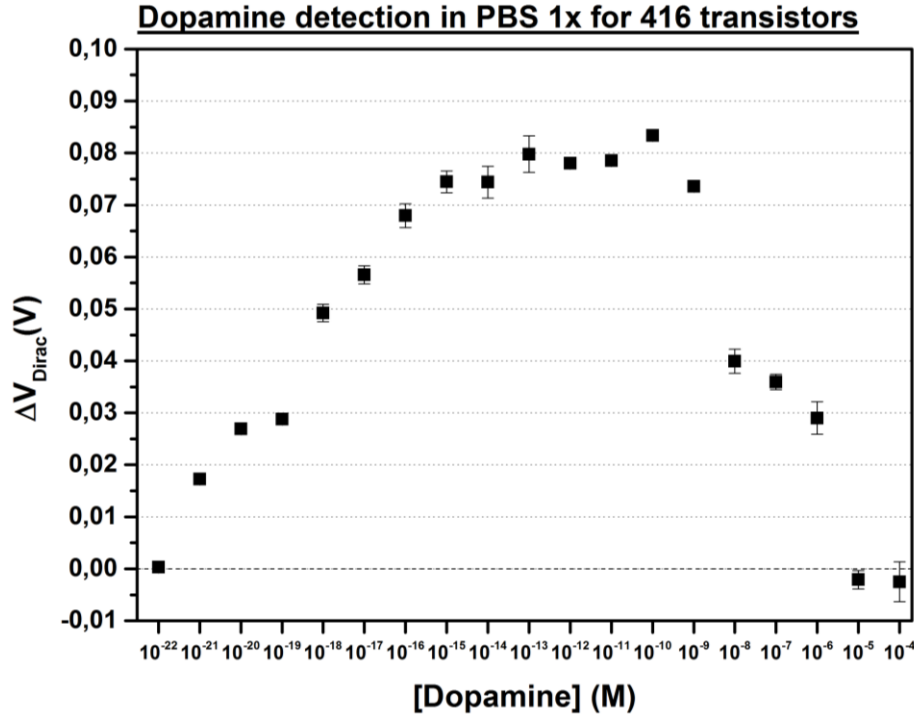


Figure 37. Shift in Dirac point (ΔV_{Dirac}) as a function of dopamine target concentrations in PBS 1x.

The first stage of detection starts on 0.1 zeptomolar (10^{-22}) and ends by 10 zeptomolar (10^{-20}). For the first concentration, we calculated, using the Avogadro constant, that there are no dopamine molecules present in the 20 μ L volume. Thus, there is no shift in the Dirac point.

For 1 (10^{-21}) and 10 zeptomolar concentrations, although we have a +17 mV and +27 mV shift, we also determined that there are no molecules for these concentrations for the given 20 μ L volume. However, we cannot explain this initial positive V_{Dirac} shift from 1 zM to 10 zM and thus, we attribute the cause to measurements' artefacts. And the 0.1 attomolar (10^{-19}) concentration has only a +2 mV shift from the previous one, which is half the error's value (4 mV, see chapter 4), meaning that this concentration's voltage shift cannot be considered as dopamine detection.

Therefore, the limit of detection (LOD) was considered to be the 1 attomolar (10^{-18}) concentration. The calculated number of dopamine molecules for this concentration in a 20 μ L droplet was 12. With a V_{Dirac} shift of +50 mV, we were able to detect a very reduced number of dopamine molecules with great sensitivity. In fact, this is the lowest LOD ever reported for dopamine detection by a factor of 3. Additionally, it should be highlighted that this LOD was achieved with a label-free method as opposed to the current state-of-the-art LOD.

The attomolar detection sets the start of our linear dopamine detection trend, this second stage is characterized by a linear detection behavior with a constant increase of V_{Dirac} as dopamine

concentrations increase until surface saturation occurs at 1 picomolar (10^{-12}) concentration. The saturation occurs at a +80 mV increase from the baseline, however since the first concentration to be detected had already a shift of +50 mV, this means that our linear range has a response of 40 mV.

Figure 38 shows the graphene transfer curve for different dopamine concentrations.

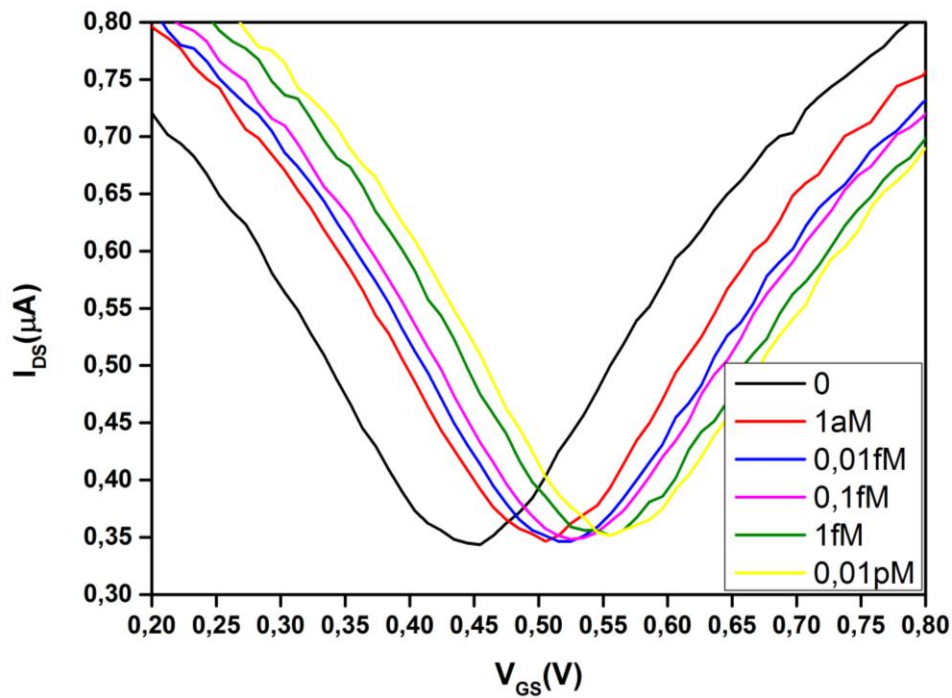


Figure 38. Exemplificative transfer curve for one transistor for different dopamine concentrations in PBS 1x.

This detection method is based on positive linear increases of Dirac point, and such can be explained by the aptamer's conformational change every time it binds to dopamine.

Before binding to dopamine, the aptamer is fully stretched and away from graphene, the aptamer's segment is about 16 nm, only about 1 nanometer of its length is within the Debye length. By changing structural conformation upon dopamine binding, more charges fall within the Debye length and contribute to the observed changes in the transfer curve Dirac point.

The electrolyte's monovalent cations, like potassium and sodium are attracted to the aptamer's phosphate-deoxyribose backbone because it has a single-bounded oxygen group available. With these stable bounds formed, Na-O and K-O, between the cations and the DNA, the overall charge of the DNA becomes less negative, however we believe that this electrolyte's cations don't have enough strength to alter the aptamer's conformation, and so, it is only altered by the dopamine binding.

Initially we have two sets of separate electrical double layers. The first EDL is composed by the graphene counter-ions, which are anions like chloride because our graphene is p-doped (it has more positive charges). The second EDL is formed around the aptamers, where the counter-ions are cations like potassium and sodium, because of DNA's negative backbone.

We hypothesized that once dopamine binds to the dopamine-specific aptamer, the aptamer changes its conformation by wrapping around itself, coming closer to the graphene channel. With this surface alteration we have the aptamers close to graphene, which means we have more negative charges stacked to graphene's electrical double layer. Consequently, for the same Debye length, we have more negative charges which increases the screening effect of positive charges on the graphene, producing a strong local gating effect. The positive charges induced on graphene are provided by the gold source and drain contacts to which graphene is connected to (see chapter 4). This detection mechanism is clarified in figure 39.

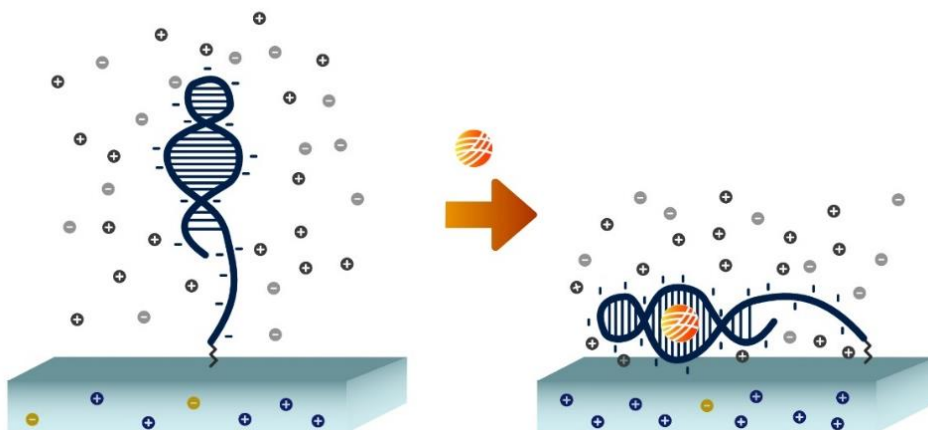


Figure 39. Exemplificative illustration of aptamer's conformational change with dopamine binding in PBS 1x electrolyte solution. On the left, the aptamer is fully stretched in PBS 1x without dopamine and the graphene is mostly p-doped. On the right, dopamine was diluted in PBS 1x and the aptamer binds to it changing its conformation and coming close to graphene which becomes more p-doped.

The linear stage of dopamine detection sees a linear increase of ΔV_{DIRAC} with increasing concentrations of dopamine. This is expected every time there is a dopamine concentration increase, there are more aptamers changing their conformation and getting close to graphene. Therefore, we have more negative charges on the electrical double layer which increases graphene's screening effect of positive charges.

The third stage of our calibration curve starts from 1 pM to 0.1 nM (10^{-10}), where surface saturation occurs, meaning that there is a lack of Dirac point shift despite the addition of higher concentrations of dopamine. This can occur due to two different scenarios, either there are no

more aptamers for dopamine to bind to, meaning that we have reached aptamer saturation, or, surface saturation has occurred, where there is dopamine binding to the aptamers but there is not space left for them to change their conformation and come close to graphene. In either case, all the negative charges are already in the electrical double layer next to graphene within the Debye length and no more shifts of the Dirac curve are observed.

The last part of the calibration curve is characterized by a negative Dirac point shift, starting from the 1 nM (10^{-9}) to 0.1 mM dopamine concentration. Since we had previously reached either aptamer or surface saturation, we hypothesized that this observed negative response of -80 mV could be due to non-specific binding of dopamine to graphene. These results point to aptamer saturation as the most likely event that's occurring for high dopamine concentrations.

Considering that 40% of graphene is not passivated (see chapter 5), dopamine being a positively charged molecule would produce a negative screening effect by directly binding to graphene and a consequent negative Dirac point shift. This would more likely occur through direct charge transfer making graphene become less p-doped. To further investigate this effect and provide support to our hypothesis, several experiments were undertaken and are described in the next sub-chapter.

6.2. Dopamine and graphene direct interaction

The observation that high concentrations of dopamine produced a negative shift of the gate voltage in our sensors, lead to the hypothesis that after aptamer saturation dopamine was directly binding to non-passivated graphene in the transistors channel. To test this hypothesis, we measured Dirac point shifts as a function of high dopamine concentrations in bare graphene and in different steps of the passivation/functionalization process. Additionally, we also tested our sensors response to high concentration of dopamine after blocking graphene with polysorbate 20 (Tween 20). Figure 40 shows results of dopamine response as function of ΔV_{DIRAC} for different surface passivation's.

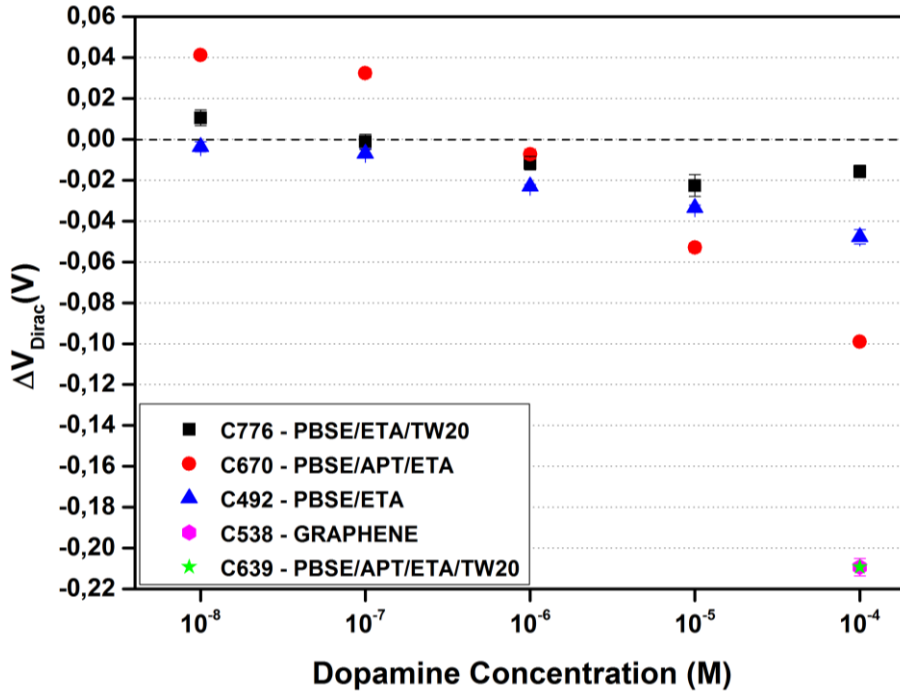


Figure 40. Shift in Dirac point (ΔV_{Dirac}) as a function of dopamine target concentrations in PBS 1x for different functionalization methods: bare graphene (pink), PBSE + ETA (blue), PBSE + APT + ETA (red), PBSE + ETA + TW20 (black) and PBSE + APT + ETA + TW20 (green).

Our label-free dopamine-specific aptamer functionalization method (red data on figure 40), where we have the negative Dirac point shift trend that cannot be explained by local gating of the aptamer conformational change. For 0.01 μM (10^{-8}) we still have a positive Dirac point shift regarding the baseline, because we started the experiment for high dopamine concentrations, meaning that the aptamers were dopamine-free and still bounded to it producing +40 mV shift. But for the next concentration, 0.1 μM (10^{-7}) we already see a decrease of V_{Dirac} of -10 mV. The sensitivity of this chip was of 140 mV.

To assess if this n-doping of the graphene was related to the aptamer-based detection or any additional interaction between non-bonded dopamine and surface aptamers, we removed the aptamer probe and incubated dopamine on a graphene channel functionalized with the blocked linker, i.e. PBSE bound to ethanolamine. Considering that PBSE only covers approximately 60% of the graphene surface, this means that exposed graphene was present. The progressive addition of higher concentration of dopamine also lead to an overall Dirac point shift of -50 mV (blue markers in figure 40). The first two concentrations did not produce a positive shift, but the response remained at the baseline value which is consistent with the fact that aptamers in this electrolyte give a positive shift when bounded to dopamine. The difference between the range of response

between both cases, with and without aptamer, is 50 mV, we were expecting to have a bigger response for the passivation without the probe but that was not the case. One possible theory is that the PBSE-ethanolamine binding occupies more area close to the graphene making it more difficult for dopamine to reach graphene.

To further confirm our hypothesis that the observed shift is a product of dopamine directly interacting with graphene, we incubated a high concentration of dopamine (0.1 mM) in bare graphene, which resulted in a Dirac point shift of -210 mV. This was the highest observed response and 4 times higher than that observed for the previous experiment with PBSE and ETA. Since the transistors had all graphene in the channel exposed to the solution, and that other molecules involved in the functionalization process were not present, this experiment suggests that the observed negative shift is likely mediated by a direct interaction of dopamine and graphene.

To test if we could block this negative shift response, we added polysorbate 20 (Tween 20) [Sigma-Aldrich] to passivate exposed graphene after PBSE and ETA functionalization steps (black markers in figure 40). Tween 20 is a non-ionic surfactant that strongly interacts with graphene [92]. It is composed of three chemical parts: aliphatic ester chains that prevent non-specific binding of biomolecules, three-terminal hydroxyl groups that are hydrophilic and an aliphatic chain that can easily be adsorbed on a hydrophobic surface like graphene by non-covalent interaction [93].

A previously reported graphene oxide sensor with aptamers combined with Tween 20 showed that the surfactant protected the graphene from cocaine's non-specific binding [90]. Another graphene biosensor passivated with Tween 20 showed bacteria non-specific binding on graphene could be prevented [91].

The observed response had a linear range of 30 mV for all the incubated concentrations, 60 mV less than a complete functionalization process without Tween 20. This indicates that perhaps Tween 20 is able to block dopamine from reaching graphene.

Although tween 20 contains aliphatic ester chains which may prevent dopamine molecules getting closer to the graphene channel, there was still a response of -15 mV for 0.1 mM of dopamine concentration. This is probably because the used concentration of Tween 20 was not enough to block all the graphene or electrostatic effects might be happening. Nevertheless, these results support our hypothesis that the negative shift observed for higher dopamine concentrations is due to a direct interaction of dopamine and graphene. However, this experiment cannot answer the question if this interaction is due to a binding process between dopamine and graphene or due to electrostatic interaction by proximity.

Dopamine detection on graphene + DDT surface and graphene + DDT + PBSE surface, was also studied (data not shown) and both produced positive Dirac point shifts reinforcing the theory that dopamine is indeed binding to graphene.

Considering that Tween 20 was able to block or at least reduce the response of dopamine directly interacting with graphene, we considered using it as blocking agent in our functionalization process.

6.3. Dopamine detection with Tween 20 functionalization

Our observations that after aptamer saturation a Dirac point shift with a linear response occurs in the opposite direction of aptamer-mediated dopamine detection, and that this response inversion is likely mediated by direct interaction of dopamine and graphene in the transistor, raises additional questions. First, that these two apparently competing processes may reduce our responses to aptamer detection and thus reduce our sensors overall sensitivity. And second, that if dopamine can interact with graphene, other molecules may do the same producing non-specific effects, thus reducing our sensors selectivity. Since Tween 20 was able to block or reduce the direct interaction of dopamine with graphene, we added Tween 20 to our functionalization process after completing the PBSE, aptamer and ETA steps. 0.1% Tween 20 diluted in PBS 1x was incubated for 1 hour after the ETA step. Figure 41 shows dopamine detection for this passivation method.

There was no dopamine detection for any concentration tested with the maximum response being of about 10 mV from 0.1 aM to 1 nM. But the negative behavior of dopamine for higher concentrations still prevailed, displaying a response of -220 mV for 100 μ M.

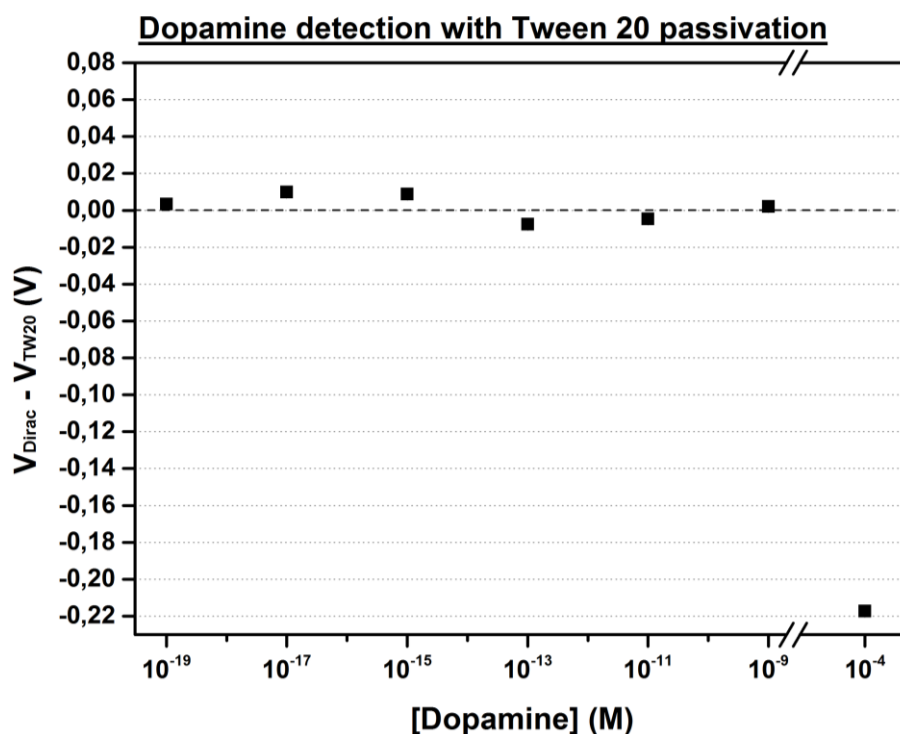


Figure 41. Shift in Dirac point (ΔV_{Dirac}) as a function of dopamine target concentrations from 0.1 aM to 100 μ M for a complete functionalization method plus Tween 20 passivation.

We believe that the lack of the aptamer-based response was due to the fact that there was no space for the aptamer to change its conformation and come closer to the graphene, because of the presence of Tween 20's aliphatic ester chains, which are almost half the length of our aptamers. However, we have not further explored this hypothesis. The blocking of exposed graphene, i.e. that did not bind to PSBE, in our sensors without interfering with the aptamers remains an open challenge. Additional blocking agents such as polyethylene glycol molecules may be tested in the future. Improving the functionalization process to increase PSBE coverage may also be a promising route.

To prove that the cause of these results was the lack of conformational change of the aptamer, we decided to remove the aptamer from the functionalization process. And so, we detected dopamine on a chip with a functionalization process based on gold blocking by DDT, PSBE linking, PSBE blocking by ethanolamine and finally graphene blocking with Tween 20. Such functionalization method can be found on figure 42.

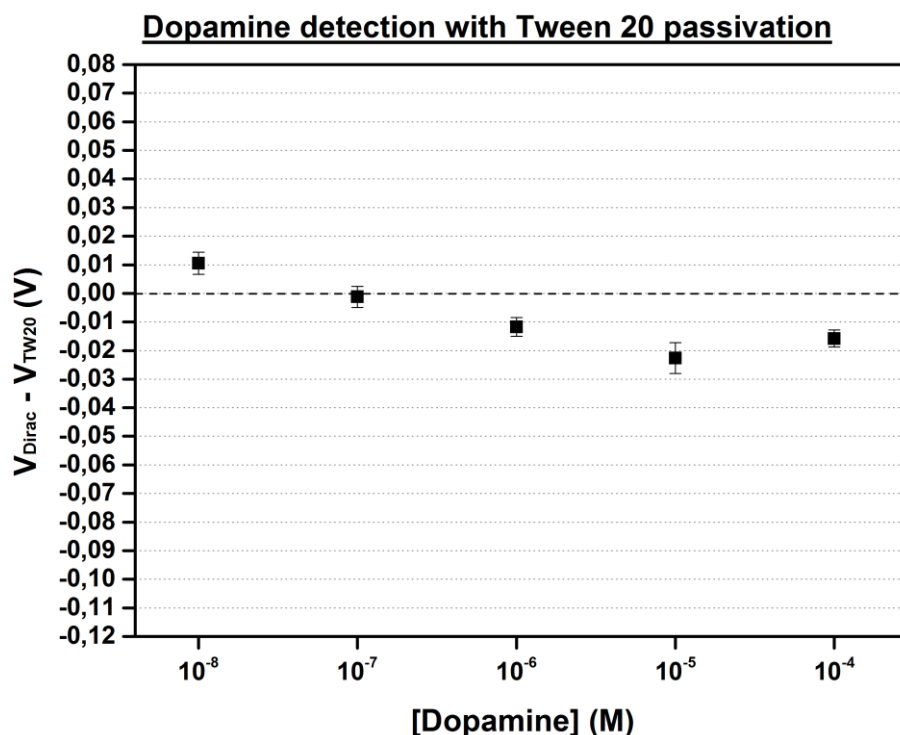


Figure 42. Shift in Dirac point (ΔV_{Dirac}) as a function of dopamine target concentrations from 0,01 μ M to 100 μ M for a functionalization method without aptamer and with Tween 20 passivation.

The sensors' average response to dopamine for the highest concentration, 100 μ M, was of -16 mV, which is not comparable to dopamine's response on graphene, -210 mV, and so, we consider there is no detection. We can then conclude that Tween 20 did indeed block the graphene surface to any other non-specific binding of dopamine, however its presence on the surface apparently may be reducing the necessary space for the aptamer to change conformation, leading to lack of detection via the aptamer's local gating.

In conclusion, non-PBSE bonded graphene blocking still remains a problem to be solved perhaps with different obstructing agents like a polyethylene glycol molecule.

6.4. Dopamine analogues and interfering agents' detection

Selectivity is very important aspect of biosensors because they must be able to detect the target of choice in very complex mediums where other molecules can behave similarly to the target in question.

Dopamine is a neurotransmitter with many analogues and interfering agents, and so it is necessary to test our detection method's selectivity to dopamine in regard to these molecules. It is necessary to understand if these molecules bind to the aptamer or to the exposed graphene channel.

L-Dopa is the direct precursor of dopamine and can be synthesized directly from L-Tyrosine. The only difference from dopamine being the lack of the amine structure.

L-Dopa was diluted in PBS 1x and a 20 μ L droplet was incubated on the chips for two hours, being measured afterwards by thirty transistors for four concentrations, 1 aM (10^{-18}), 1 fM (10^{-15}), 1 pM (10^{-12}) and 1 nM (10^{-9}).

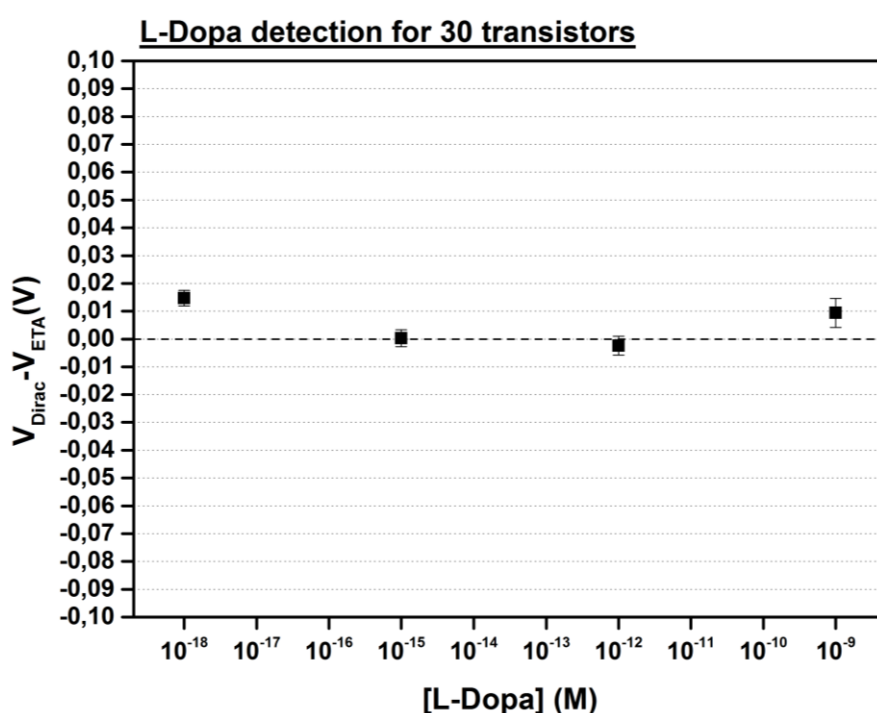


Figure 43. Shift in Dirac point (ΔV_{Dirac}) as a function of L-Dopa target concentrations for 30 transistors.

The results show that there was a +15 mV response on the attomolar level but on the nanomolar concentration it was about +10 mV.

Furthermore, we also tested our sensors' specificity to other analogues and interfering agents like L-Tyrosine, Homovanillic Acid and Ascorbic Acid.

L-Tyrosine is the precursor for L-Dopa which is synthesized into dopamine. This amino acid was diluted in PBS 1x and incubated on thirty-five transistors for two hours for each concentration of L-Tyrosine, of 1 pM (10^{-12}), 1 nM (10^{-9}) and 1 μ M (10^{-6}). For the first two concentrations, there was no response and for the micromolar it was only of +10 mV.

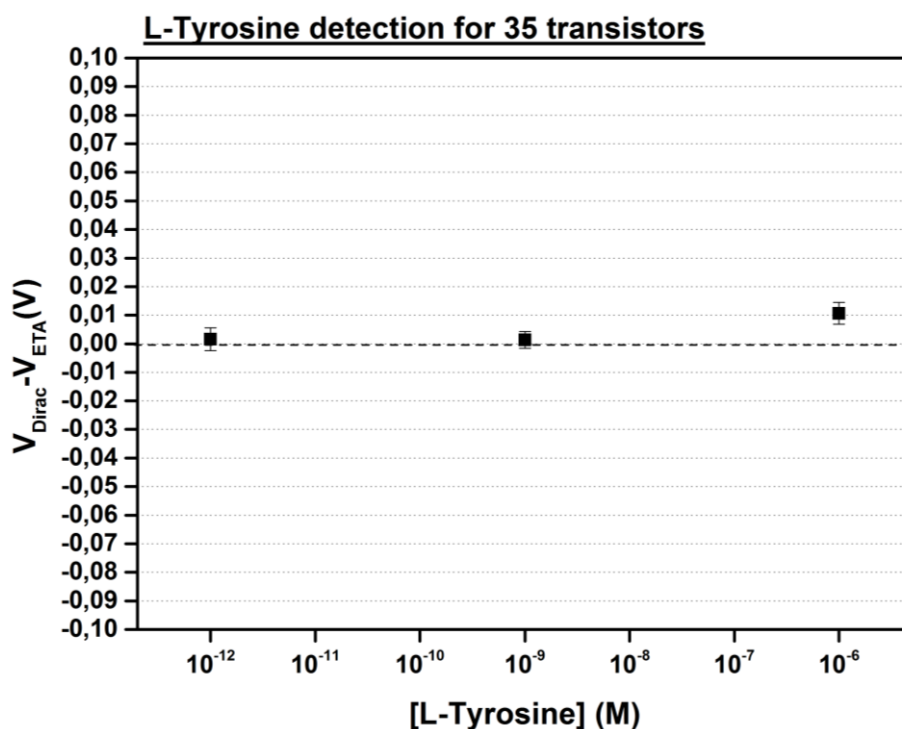


Figure 44. Shift in Dirac point (ΔV_{Dirac}) as a function of L-Tyrosine target concentrations for 35 transistors.

And then, we also tested if different acids like Homovanillic Acid or Ascorbic Acid would interfere with the measurements by also diluting each in PBS 1x and incubating them for two hours.

Homovanillic acid is a molecule from dopamine's direct pathway. It is a catecholamine metabolite that is produced by the consecutive action of monoamine oxidase and catechol-O-methyltransferase on dopamine. Homovanillic acid is used as a reagent to detect oxidative enzymes and is associated with dopamine levels in the brain.

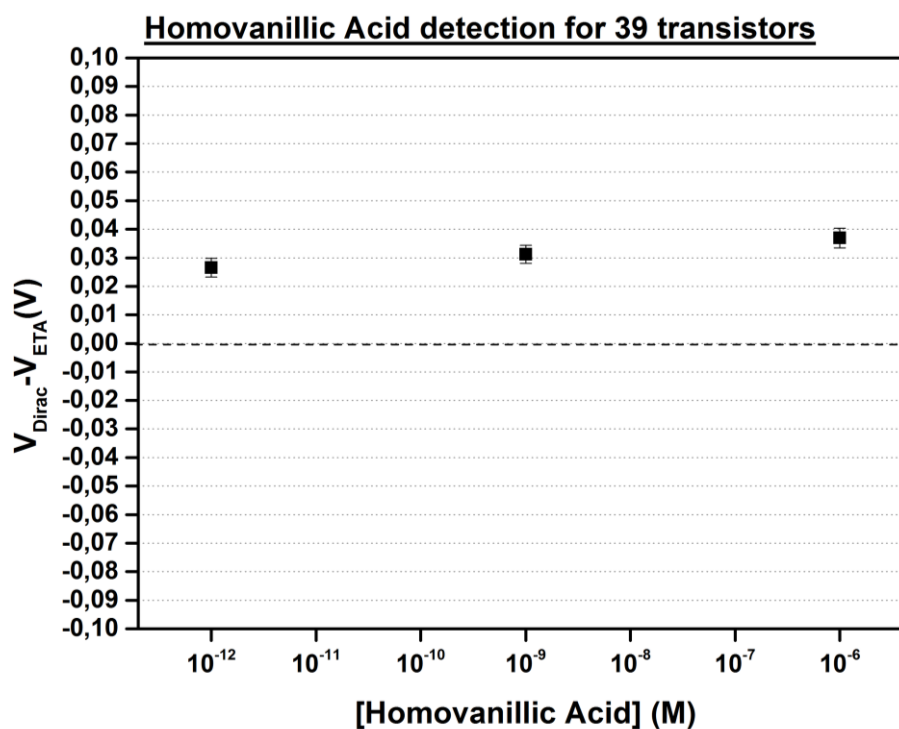


Figure 45. Shift in Dirac point (ΔV_{Dirac}) as a function of Homovanillic Acid target concentrations for 39 transistors.

Homovanillic Acid had a more considerable response compared to the previous experiments, having an average of +30 mV Dirac point shift for all incubated concentrations.

Ascorbic Acid, also known as vitamin C, is an essential nutrient responsible for tissue repair and the enzymatic production of certain neurotransmitters. It is present on the extracellular medium in concentrations higher than the neurotransmitters and its redox potential is relatively close to of the other neurotransmitters which causes interference with voltametric methods. Therefore, Ascorbic Acid is commonly used as an interfering agent in neurotransmitters biosensing.

For thirty-six transistors we had an average response of +19 mV for every concentration.

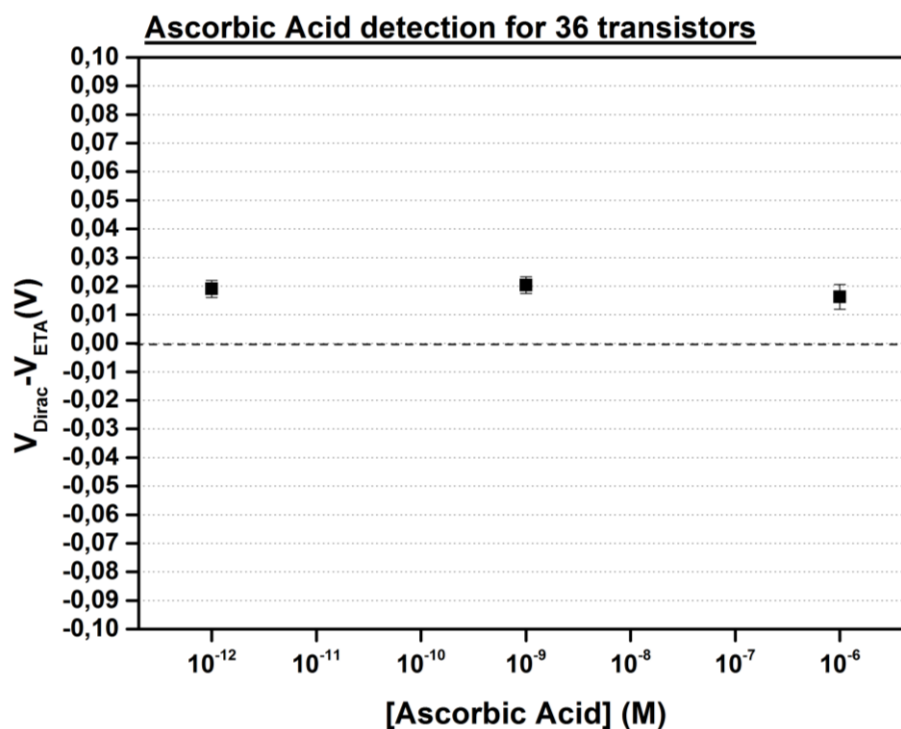


Figure 46. Shift in Dirac point (ΔV_{Dirac}) as a function of Ascorbic Acid target concentrations for 36 transistors.

L-Dopa's very low response or lack of it for the femtomolar and picomolar concentrations proves that the aptamer and the functionalization process are very selective to dopamine only. For L-Tyrosine the biggest response was of only 10 mV.

In figure 47 we have dopamine detection on the femtomolar concentration with a response of +100 mV, whereas L-Dopa gave a -4 mV response for 1 micromolar, despite being 9 times more concentrated than dopamine still gave a negligible response.

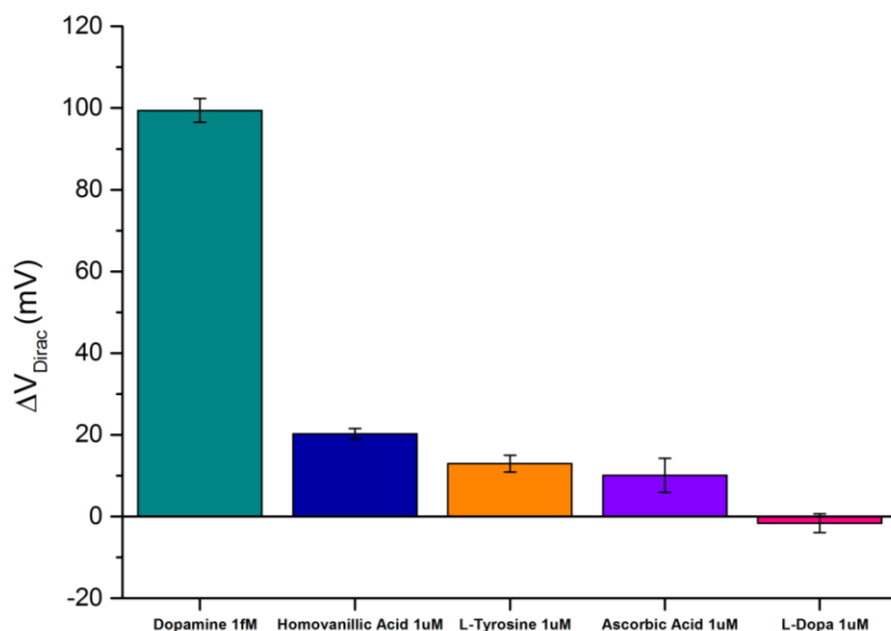


Figure 47. Comparative graph of dopamine's response with its analogues and interfering agents' response.

Both Homovanillic Acid and Ascorbic Acid had a considerable positive response of around 20 mV that should be taken into account for more complex medium experiments.

However, for the two amino acids precursors of dopamine, which are structurally very similar to dopamine, we barely observed any detection which is indicative of our sensor's specificity. Our good results are derived by the fact that we used a highly selective biorecognition element method and avoided redox reactions typically used in electrochemical and catalytic detection methods [1], since dopamine and its analogues have similar redox potentials making it difficult to tell apart the neurotransmitters. Nevertheless, some electrochemical dopamine sensors were shown to be more selective to dopamine by modification of the electrodes with nanomaterials and affinity ligands [10], [51], [47], including with graphene [49].

A liquid crystal biosensor for dopamine with signal amplification through functionalization of gold nanoparticles, selectively detected dopamine between molecules with structural similarities such as ascorbic acid, glucose, epinephrine, norepinephrine, tyramine and serotonin but the limit of detection was on the micromolar dopamine concentration [51].

Other dopamine sensors based on the existing RNA [18] or DNA [15] dopamine-specific aptamers also reported high selectivity [16] [105], although it has also been reported that the DNA conversion of the RNA aptamer is not selective against other catecholamine neurotransmitters [104]. In this work we did not test other neurotransmitters from the monoamines groups, such as

serotonin and norepinephrine. However, Nakatsuka, Nako, *et al.* [15] showed that the same dopamine-specific DNA aptamer was highly selective when compared to these neurotransmitters and 5-HIAA, serotonin's metabolite.

Some different works on dopamine detection with a selective method but different recognition elements were also reported.

For example, biosensors based on the enzyme tyrosinase have been successfully used for selective determination of dopamine in the presence of ascorbic and uric acids in a biological environment. But the main problem in the practical application of such biosensors is their poor long-term stability and reproducibility [50].

Maciejewska, J., *et al.* [75] developed a tyrosinase modified electrode, and demonstrated that the presence of these interferents does not affect the selectivity. However, this amperometric detection of dopamine lacked a low LOD (0.1–0.5 μM). Another amperometric biosensor, based on tyrosinase immobilized on a boron-doped diamond electrode, was also able to selectively detect dopamine but with a high LOD of 1.3 μM in presence of ascorbic acid [72].

6.5. L-Dopa effect on passivation without aptamer

To test if the dopamine is in fact binding to exposed graphene, we also used L-Dopa as a negative control in an additional experiment. Increasing concentrations of L-Dopa, from 0.01 μM to 0.1 mM were added in 20 μL droplets to a chip without aptamer but passivated with the blocked linker (i.e. PBSE + ETA). Figure 48 shows L-Dopa detection without aptamer.

As L-Dopa is chemically similar to dopamine, only lacks the amine structure, we detected it on a chip without aptamer, functionalized only with the blocked linker, PBSE-ethanolamine.

With this experiment we wanted to analyze what effect a molecule identical to dopamine would have on graphene. If we don't have the negative voltage effect that is present on the dopamine detection for high concentrations, it can indicate that it's the amine group that is indeed binding to graphene.

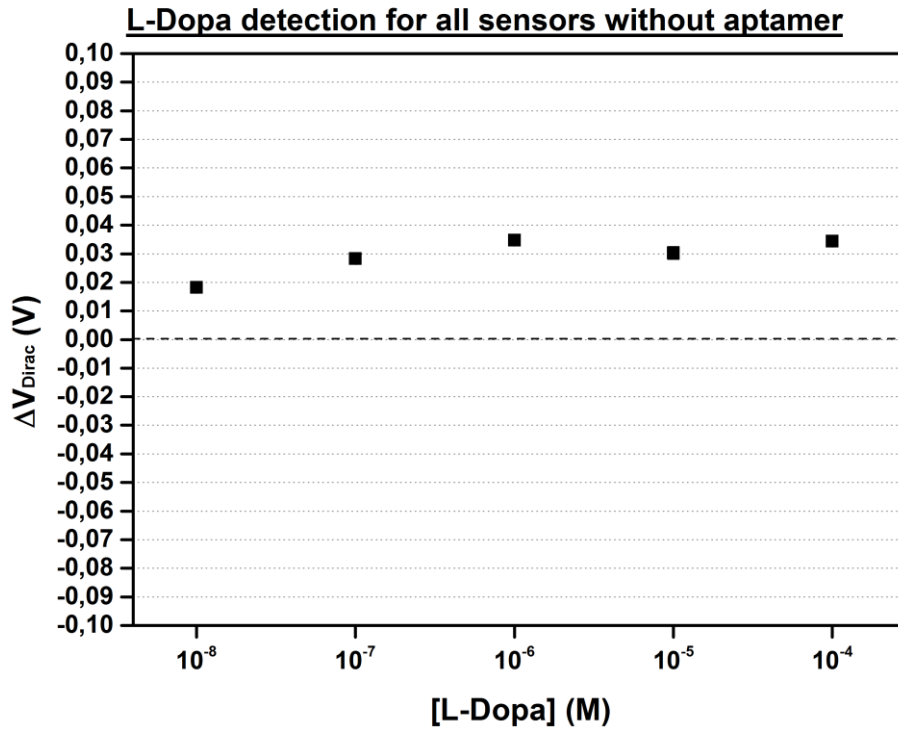


Figure 48. Shift in Dirac point (ΔV_{Dirac}) as a function of L-Dopa target concentrations without aptamer.

We observed a positive Dirac point shift with increasing concentrations of L-Dopa. This average response of +30 mV from the baseline, was the opposite of the negative response observed for high concentrations of dopamine (see subchapter 6.1.). This result suggests that dopamine may be binding to graphene through the amine group [98], since L-Dopa is chemically similar to dopamine, only lacking the amine group.

6.6. Dopamine detection in artificial cerebrospinal fluid (aCSF)

In order to transition our *in vitro* detection to an *ex vivo* setting, first, we need to validate our functionalization process on a more complex electrolyte medium, such as artificial cerebrospinal fluid (aCSF).

aCSF is a buffer solution prepared with a composition representative of cerebrospinal fluid (CSF, a clear fluid that surrounds the brain and spinal cord). aCSF is used experimentally to immerse isolated brains, brain slices, or exposed brain regions to maintain osmolarity, and to buffer pH at biological levels and commonly utilized for electrophysiology experiments to maintain the

neurons that are being studied. The reason why we use aCSF is because it is the closest extracellular medium to biological CSF.

Fresh aCSF was prepared whenever experiments took place. aCSF solution is composed by 127 mM NaCl, 1.0 mM KCl, 1.2 mM KH_2PO_4 , 26 mM NaHCO_3 , 10 mM D-glucose, 2.4 mM CaCl_2 and 1.3 mM MgCl_2 .

The following graph shows our dopamine detection in aCSF electrolyte for 54 transistors from 0.1 aM to 1 nM concentrations and figure 50 shows graphene transfer curve for the same dopamine concentrations.

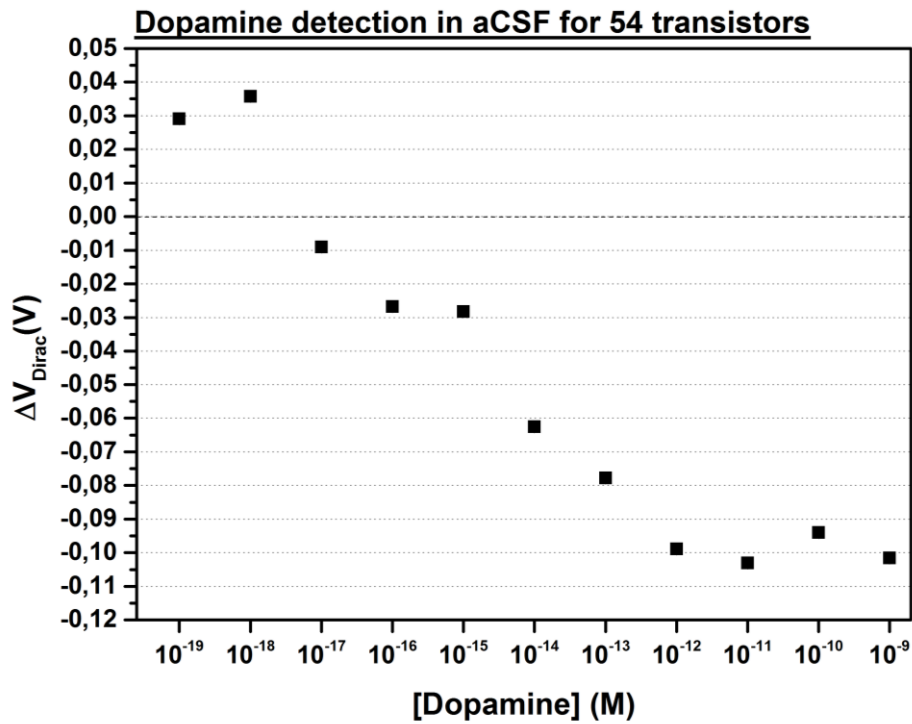


Figure 49. Shift in Dirac point (ΔV_{Dirac}) as a function of Dopamine target concentrations for 54 transistors in aCSF medium.

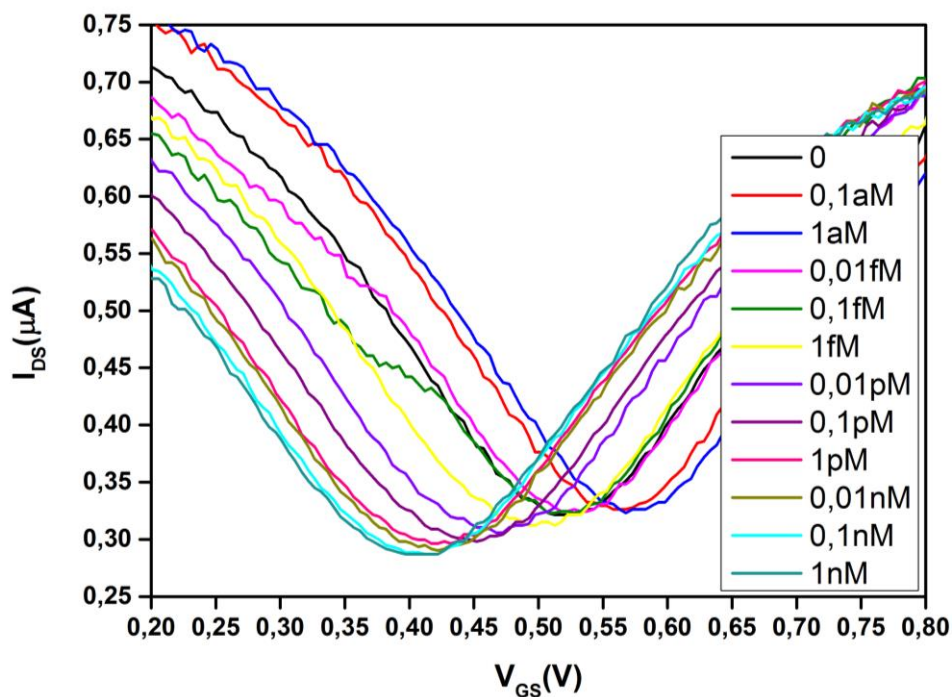


Figure 50. Graphene transfer curve for different dopamine concentrations in aCSF 1x.

For the first two concentrations, 0.1 aM and 1 aM, we have a Dirac point shift of +30 mV and +35 mV respectively. One of the reasons for these concentrations to have a positive response whereas higher dopamine concentrations have negative response, could be because for low dopamine concentrations the negative backbone of the DNA dictates the electrical double layer's charges, while for higher concentrations the electrolyte's interaction with the aptamer can no longer be neglected. Another possibility is that this positive shift is caused by measurements' artefacts that we can not explain as of now. Therefore, we should exclude 0.1 aM and 1 aM as dopamine detection, making the 10 aM the limit of detection with a Dirac point shift of -45 mV in respect to the previous dopamine concentration. Since this electrolyte is much more complex than PBS, we were expecting to have a higher limit of detection, but even so, it is still lower than any previously reported LOD for dopamine detection in any electrolyte. Figure 50 is an exemplificative illustration of aptamer's conformational change in aCSF 1x.

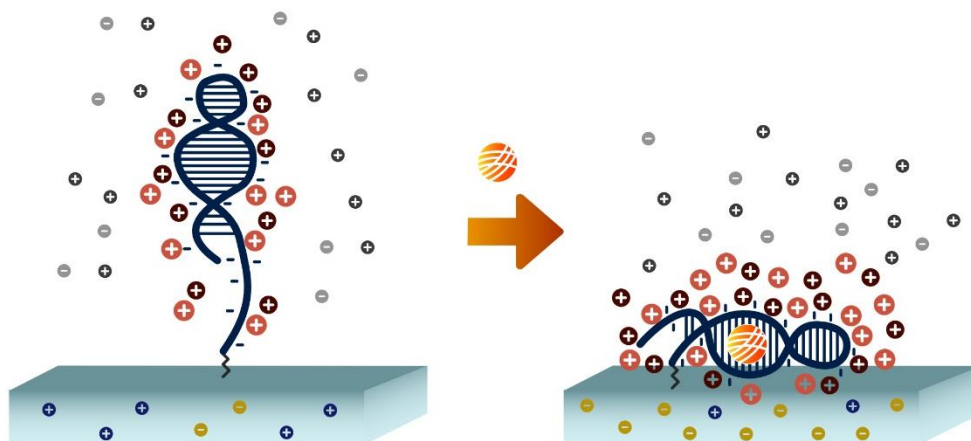


Figure 51. Exemplificative illustration of aptamer's conformational change in aCSF 1x electrolyte solution. On the left, the aptamer is fully stretched in aCSF without dopamine and the graphene is mostly p-doped. On the right, dopamine was diluted in aCSF and the aptamer binds to it changing its conformation and coming close to graphene which becomes less p-doped due to divalent cation aptamer enhancement.

A surprising observation was that the response of our sensors to dopamine in aCSF was different from the one observed in PBS. While PBS had a positive linear response with increasing dopamine concentration (p-doping), in aCSF we observed the exact opposite behavior, negative linear response (n-doping). And so, we tried to justify this difference considering the electrolyte's compositions.

One of the main differences between PBS and aCSF is that the latter contains divalent cations (Mg^{2+} and Ca^{2+}) instead of monovalent cations (K^+ and Na^+). Divalent cations have bigger electrostatic interactions when compared to monovalent ions, which affect cooperative and competitive effects of counter-ions in solution [97].

DNA's backbone has one possible binding site, a single bounded oxygen from the phosphate group, meaning that magnesium and calcium ions will bind to it, but since they are divalent, another possible single bound can be established. Mg^{2+} and Ca^{2+} can either attach to the next phosphate chain or remain available. Both elements have more atomic mass than sodium and potassium, meaning that not only they are heavier which can affect the aptamer's conformational change but also, the electrical double layer composed of these counter-ions will stack on graphene's EDL overshadowing the aptamer's negative backbone. The EDL is then composed of positive charges meaning that the screening effect on graphene will produce negative charges.

This binding of divalent cations to the phosphate group of the aptamer's backbone theory has been similarly explained by Korolev, Nikolay, *et al.* [94]. But the cations can also just be electrostatically imprisoned to the aptamer.

Another possibility is that the presence of divalent cations changes the conformation of the aptamer even before it binds to dopamine, having for example, the aptamer close to the graphene before finding its target, producing different changes in surface charge rearrangement on the graphene channel. Such has been reported by Nakatsuka, Nako, *et al.* [96], showing that this dopamine aptamer exhibits different target binding affinities in presence of divalent cations since they influence the structure of the dopamine aptamer-target complex. Thus, they hypothesized that different aptamer conformational changes occur upon dopamine binding in the presence vs absence of the divalent cations.

At 0.1 nM dopamine concentration surface saturation occurs at a Dirac point shift of -100 mV. The linear range of dopamine detection in aCSF is 140 mV, 60 mV more than detection in PBS 1x, surface saturation occurs two concentrations higher than PBS'. Like previously reported, higher receptor-target affinities and bigger response occur in the presence of divalent cations with this specific aptamer [95] [96].

This different detection response for the aCSF electrolyte solution allows us to better understand how our aptamer behaves on a more complex medium and confirms that it is possible to detect dopamine with great sensitivity (10 aM) in a very complex electrolyte, setting the bridge for dopamine detection in biological CSF. Detection of dopamine in biological CSF and in *ex vivo* brain samples can be found in the next chapter.

7. DOPAMINE DETECTION *EX VIVO*

For *ex vivo* experiments, cerebrospinal fluid, blood and striatum homogenate were extracted from mice. The objective of these experiments was to assess our sensor's selectivity and specificity in a very complex medium that is a real brain, in this case, a mouse brain.

Since many different molecules can be found in the brain, to guarantee that we were in fact measuring dopamine, we used a mice model where dopamine could be acutely depleted from neuronal synapses. Such model was based in injections of a monoamine-depleting drug, reserpine, that irreversibly and non-selectively blocks the vesicular monoamine transporter [99]. Reserpine inhibits the activity of the vesicular monoamine transporter 1 and 2 (VMAT-1 and VMAT-2). Since VMAT-2 occurs in neurons, reserpine impairs dopamine uptake and storage in neuronal cells in the brain, including in the striatum brain region, leading to the depletion of dopamine in neuronal synapses [100]. First, we confirmed that reserpine could in fact lead to dopamine depletion in the brain by comparing cerebrospinal fluid (CSF) extracted from reserpine injected animals and controls. Then, to confirm that we could detect dopamine in a complex solution that closely mimics the extracellular space of the brain we used brain homogenates from dopamine-depleted animals to which dopamine was added.

An additional preliminary experiment with dopamine detection in blood extracted from mice can also be found in Supplement 3.

This chapter's and previous chapter's data was presented at the Portuguese Society for Neuroscience Meeting ("SPN2021") conference (see abstract on Supplement 2).

7.1. Experimental procedures

Animal experiments were carried out at the Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, and were conducted in accordance with European Union Directive 2016/63/EU and the Portuguese law on the protection of animals for scientific purposes (DL No 113/2013). This study was approved by the Portuguese National Authority for Animal Health (DGAV, 8519) and the Ethics Subcommittee for Life and Health Sciences of University of Minho (SECVS, 01/18). All procedures in animals were performed by researchers accredited by FELASA and DGAV.

Mice (n=5) were injected with reserpine (5 mg/Kg, i.p.) dissolved in 1% glacial acetic acid and diluted in 0.9% saline.

Control mice (n=5) were injected with the vehicle (1% glacial acetic acid diluted in 0.9% saline).

8h post-administration, reserpine-injected mice displayed severe akinesia, postural instability, and tremors while control mice displayed normal behavior. Akinesia and postural instability are an indication of dopamine depletion since one major dopamine pathway, the nigro-striatal pathway, is involved in motor control. Mice were then anesthetized with avertin (tribromoethanol; 20 mg/mL, i.p.) placed in a stereotaxic frame for head fixation and CSF was extracted by cisterna magna puncture. Extracted CSF samples were immediately frozen in liquid nitrogen and stored at -80°C. Mice were then perfused transcardially with 0.9% saline to remove all circulating blood and the brain was quickly removed. The striatum, on both hemispheres, was dissected, frozen in liquid nitrogen and stored at -80°C.

Before use, brain samples were thawed, and two samples were combined in a single Eppendorf tube with aCSF added at 5 μ L/mg. Samples were then centrifuged at 14000 rpm, for 15 mins at 4°C.

7.2. Dopamine detection in CSF

CSF samples from 4 reserpine-injected and 4 control mice were thawed to room temperature before use. Each sample had an approximate volume of 2 μ L and was incubated for 10 minutes. CSF from control mice was incubated for every chip for the same time interval as reserpine-injected CSF. Pairs of reserpine/control animals' samples were incubated on the same chips under the same conditions so the only difference between the measurements would be the lack of monoamines, such as dopamine.

The chips baseline was acquired with artificial cerebrospinal fluid incubation and produced a Dirac point shift similar to our chips' natural error, around 5 mV, implying that there is barely any measurement difference between PBS 1x and aCSF 1x prior to aptamer-dopamine binding.

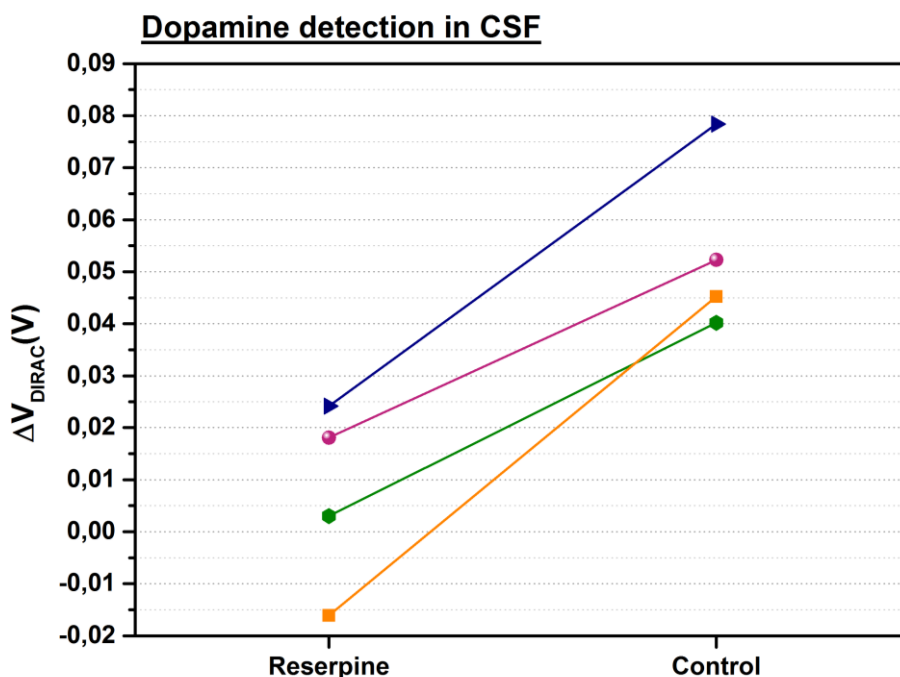


Figure 52. Reserpine-injected and control mice dopamine detection as function of Dirac point for 4 chips.

Figure 52 shows the Dirac point shift from reserpine-injected animals to control animals under the same chips. For reserpine-injected CSF, we have an average of +10 mV Dirac point shift. Control mice cerebrospinal fluid gave an average of +55 mV Dirac point shift.

The +10 mV shift from the baseline in reserpine-injected animals is most likely caused by the fact that reserpine does not deplete dopamine completely, so there might still be some dopamine present in CSF, hence the positive response observed. Such has also been verified before by Senel, Mehmet, *et al.* [101]. Nevertheless, reserpine can deplete dopamine in some brain areas up to 96% of the observed baseline levels [103].

One other reason for reserpine-injected mice giving a considerable positive shift might be because there are other molecules present in the brain, other than the depleted monoamines, that can be binding non-specifically with our graphene. Thus, this value should be considered the new baseline, doing such, the average difference from reserpine data to control measurements is of +45 mV. Such voltage shift magnitudes in aCSF dopamine detection are associated with dopamine concentrations on the femtomolar level.

Although biosensors have seldomly been used to detect dopamine in biological CSF from mice, a recent report from Senel, Mehmet, *et al.* [101] with a microfluidic dopamine-sensing device produced an amperometric response 3 times lower for reserpine treated-animals when compared with controls. This response magnitude is in line with our results, where a 5-fold difference between

dopamine-depleted animals and controls was observed. The disparity between the 3-fold difference reported by [101] and the 5-fold difference we observed, even for approximately the same sample volume, may be indicative of our sensors sensitivity to detected smaller changes in concentrations.

If we match the average response of +55 mV that we observed for control animals to the aCSF calibration curve, the total dopamine concentration measured would be in the range of 0.1 fM (10^{-16}), which is well below the 0.42 nM (10^{-10}) reported by [101]. Since the biological concentration of dopamine in CSF is not known, and that methodological variations in CSF sample extraction and preparation exist between the two works, we cannot ascertain which method was more reliable in detecting true biological concentration of dopamine in CSF.

Nevertheless, this experiment confirmed that reserpine is able to deplete most of the dopamine present in the brain which is fundamental for the following experiment with striatum homogenate.

With our voltage variation from reserpine-injected mice to control mice, we can conclude, that we are detecting dopamine in cerebrospinal fluid with our functionalization method with great sensitivity (femtomolar dopamine concentration). Initial baseline variation could be caused by the lack the selectivity of our sensors but its most likely caused by the residual presence of some dopamine concentration that was not depleted by the reserpine drug.

7.3. Dopamine detection in striatum homogenate

To assess if our sensors could detect dopamine in a highly complex solution that could closely mimic brain extracellular medium, we carried out dopamine detection experiments with striatum homogenate from mice as the electrolyte for our chips. The brain homogenate is more complex than aCSF and CSF because, it is not only composed by all the ions that exist in CSF but also brain cells, amino acids, peptides and proteins.

Following the absence, or very low levels, of dopamine in CSF we were confident that reserpine also depleted dopamine in striatal synapses. In fact, a report by McKinley, Jonathan W., *et al.* [103] confirmed through HPLC acute dopamine depletion and reduced content of striatal dopamine by 96% in striatal homogenates from reserpine-injected mice.

The mice used were reserpine-injected meaning that they had no dopamine, the objective of this experiment was to assess our functionalization process viability to detect our neurotransmitter in a very complex medium.

We choose to extract the striatum part of the brain because the striatum is densely innervated by dopaminergic synapses (from the mesolimbic dopamine pathway and from the nigrostriatal pathway as described in chapter 2). Accordingly, it is one of the brain areas with more concentration of dopamine [102]. Thus, by using animals injected with reserpine we can guarantee that dopamine will be absent from these samples, but still be able to use the homogenate of a brain area which naturally contains dopamine and would be a possible target for *in vivo* measurements.

Striatal brain samples from reserpine-treated animals were thawed, and two samples were combined in a single Eppendorf tube with aCSF added at 5 $\mu\text{L}/\text{mg}$ for homogenization with a brain tissue homogenizer. Samples were then centrifuged at 14000 rpm, for 15 mins at 4°C. Dopamine diluted in aCSF 1x (pH 7.4) was added to homogenate supernatant samples on a 1:1 volume ratio, for final dopamine concentrations ranging from 1 aM to 10 nM. Experiments were carried out in 3 chips, all had identical responses, thus only data from one is shown in figure 53.

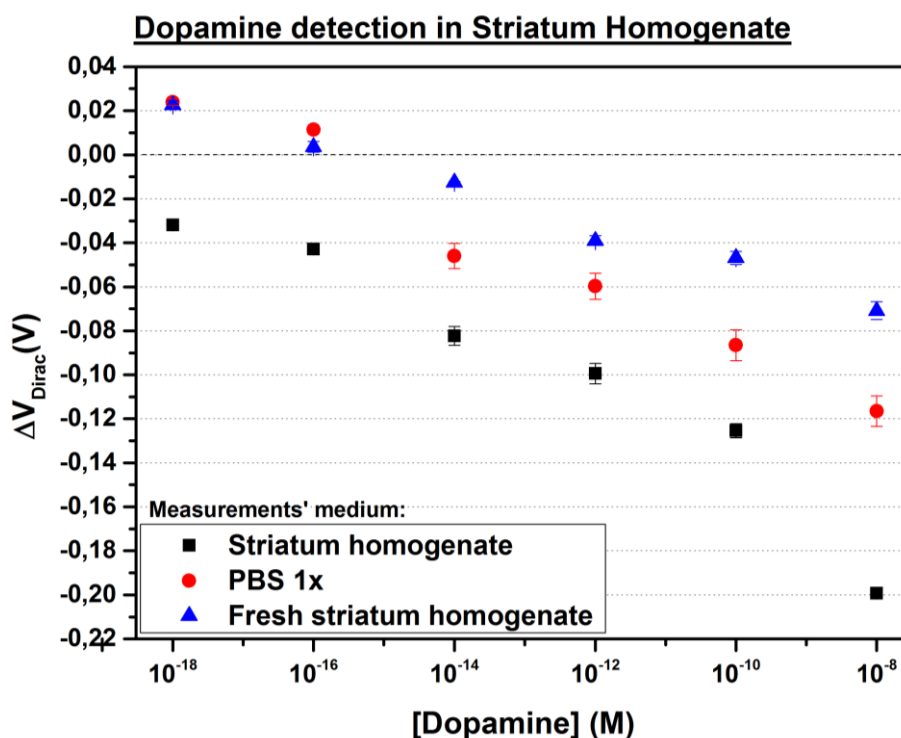


Figure 53. Dopamine detection in striatum homogenate as function of ΔV_{Dirac} . Striatum homogenate spiked with dopamine measured after 20 minutes of incubation (black), PBS 1x cleaning and measurement in PBS 1x (red) and PBS 1x cleaning and measurement in fresh striatum homogenate without dopamine (blue).

For each dopamine concentration added three different measurements of the Dirac point were taken, each in a different measuring medium. Since we did not know what signals to expect

from striatum homogenate, we measured dopamine in three distinct situations to understand which scenario detected only dopamine. We did so by washing the electrolytes with PBS 1x and incubating and measuring in dopamine-spiked striatum homogenate, in PBS 1x and fresh striatum homogenate (non-dopamine-spiked).

Initially, a baseline value was obtained with a droplet of striatum homogenate without added dopamine incubated for 20 mins. Then, a baseline of this animal's striatum homogenate before being spiked with dopamine, was obtained with the measurement of a 10 μ L droplet incubated for 20 minutes on the chip.

Then, for each dopamine concentration, a 10 μ L droplet of striatum homogenate with added dopamine was incubated on the chip for 20 minutes and the transfer curve and Dirac point were measured after this period (figure 53, black).

Afterwards, the chip was cleaned with PBS 1x and another measurement was taken with a fresh 10 μ L droplet of PBS 1x (figure 53, red). A 10 μ L droplet of striatum homogenate without added dopamine -as the one used to determine the baseline- was added to the chip and the measurements taken. All measurements displayed a negative Dirac point shift with a linear trend. This observed negative shift is similar to the one observed for aCSF (see sub-chapter 6.6).

Both PBS 1x and striatum homogenate without dopamine measurements for 1 μ M gave a positive shift of +20 mV whereas for the same concentration, the original striatum homogenate with dopamine gave a negative shift of -30 mV.

This 50 mV difference between measurements for the same dopamine incubation indicates that before cleaning the incubated medium, the chip was also measuring the dopamine effect directly on graphene and perhaps other molecules. The Dirac shift is positive for the first concentration measured in PBS 1x and non-spiked striatum homogenate, which is the same as in aCSF detection, therefore we also consider this measurement to be an artefact which makes the next concentration the LOD for striatum homogenate dopamine detection.

For the second incubated dopamine concentration, 0.1 μ M, a negative Dirac point shift was observed when compared with the previous dopamine concentration. The shift was of -10 mV for the non-dopamine spiked striatum homogenate and PBS 1x. For the striatum homogenate without dopamine the Dirac shift is -10 mV relatively to the μ M concentration and the negative behavior is kept throughout all dopamine concentrations. The same negative Dirac point trend is also found for the PBS 1x and striatum homogenate without dopamine for all concentrations.

The type of measurements that can be considered as only dopamine detection are the PBS 1x and the striatum homogenate without dopamine measurements, because for both cases the chip's surface was cleaned and the acquired data is only representative of what is left bound to the aptamers, meaning dopamine. On the first type of acquired data, striatum homogenate spiked with dopamine, not only we have dopamine detection but also electrostatic binding of other molecules to the graphene channel.

However, we can not explain the difference between PBS 1x and non-spiked striatum homogenate, since PBS 1x is a less complex electrolyte it should have given lesser response than striatum homogenate, but the overall linear range of detection for each was 130 mV and 70 mV, respectively. Dopamine detection in the incubated medium, striatum homogenate spiked with dopamine had a linear detection range of 170 mV.

The limit of detection, 0.1 fM, in striatum homogenate gave a 20 mV shift in regard to the previous concentration, whereas for *in vitro* medium, in PBS 1x the response was 70 mV and in aCSF 1x, 60 mV. With the increase of the electrolyte's complexity our sensors' sensitivity decreases, this can be caused by the many molecules that are present in the brain, that can bind by electrostatic effects to the non-functionalized graphene surface.

The linear range for PBS 1x was 40 mV, for aCSF 1x was 140 mV and for striatum homogenate measured with this buffer without dopamine was 70 mV. Our aptamer conformational change is enhanced by divalent cations, as seen on *in vitro* detection chapter, which may explain why the response is bigger than on PBS 1x (which has only monovalent cations).

Despite striatum's detection sensitivity being lower than aCSF's, our sensor's aptamers didn't saturate for the incubated concentrations, the last one being 10 nM, whereas in artificial cerebrospinal fluid saturation occurs for 0.1 nM, two concentrations lower than the non-artificial medium. However, since aptamer saturation didn't occur for the 10 nM dopamine concentration even though this medium is much more complex than aCSF, this might mean that the complex medium made it more difficult for dopamine to reach the aptamers, therefore, for the same aptamer concentration, we didn't reach surface saturation because not all aptamers bounded to dopamine, hence the lower sensitivity of striatum homogenate's measurements when compared to aCSF.

Higher concentrations of dopamine were not tested due to the low volume of homogenate samples, which only allowed testing 6 different concentrations with this protocol. Meaning that,

surface saturation wasn't reached, and perhaps higher dopamine concentrations could still be detected with this functionalization method on an *ex vivo* setting.

Figures 54 to 56 show graphene transfer curve for dopamine detection in striatum homogenate for the three types of measurements.

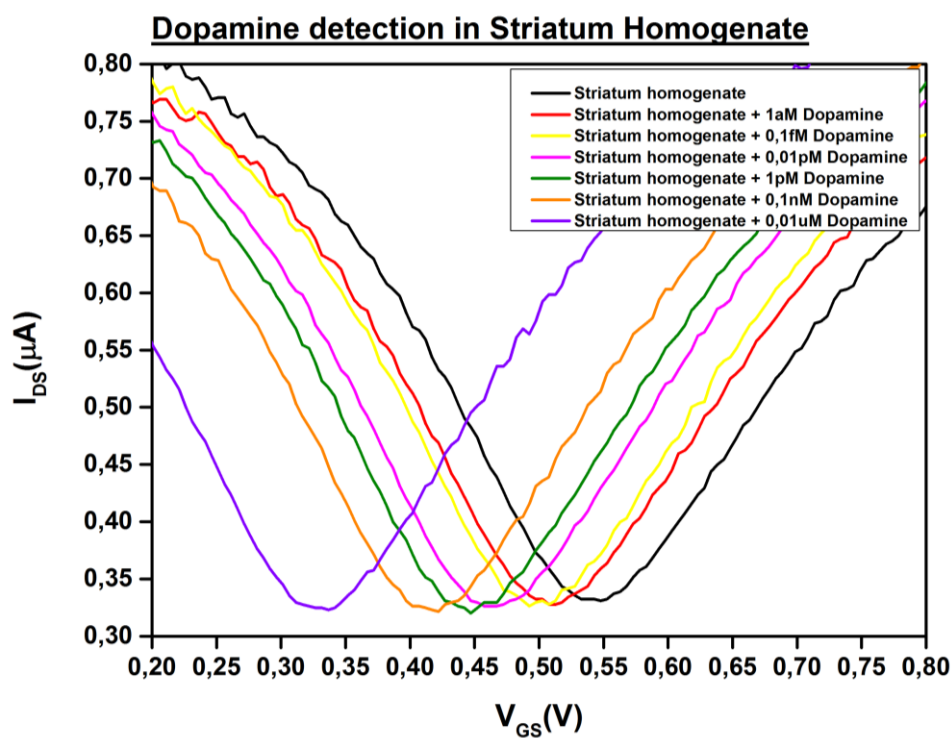


Figure 54. Graphene transfer curve for dopamine detection in striatum homogenate with measurements on the incubated electrolyte.

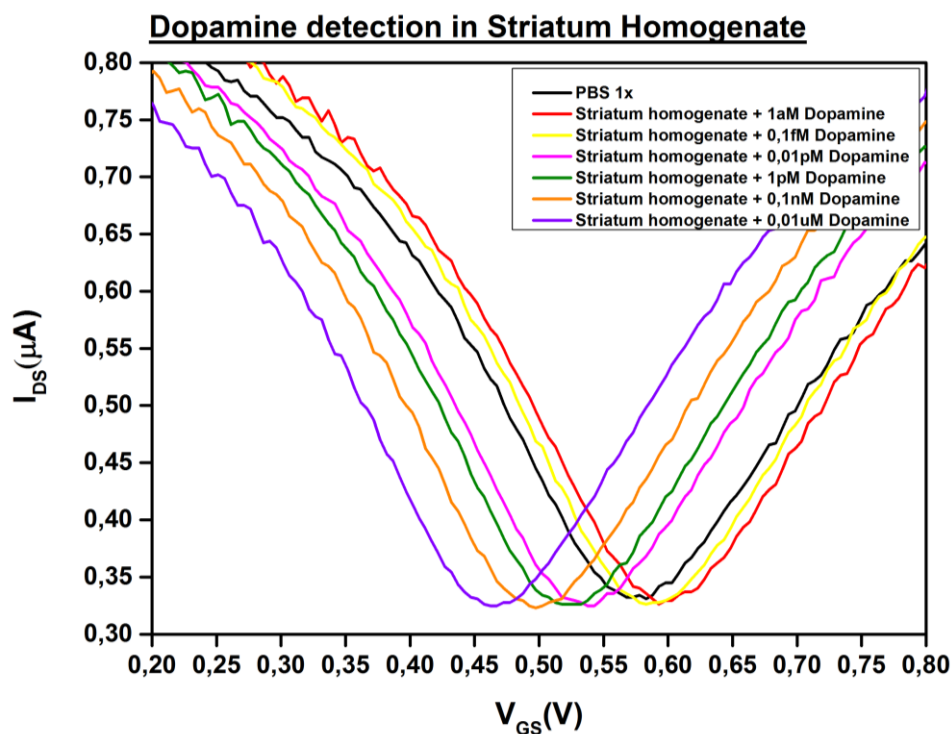


Figure 55. Graphene transfer curve for dopamine detection in striatum homogenate with measurements on PBS 1x.

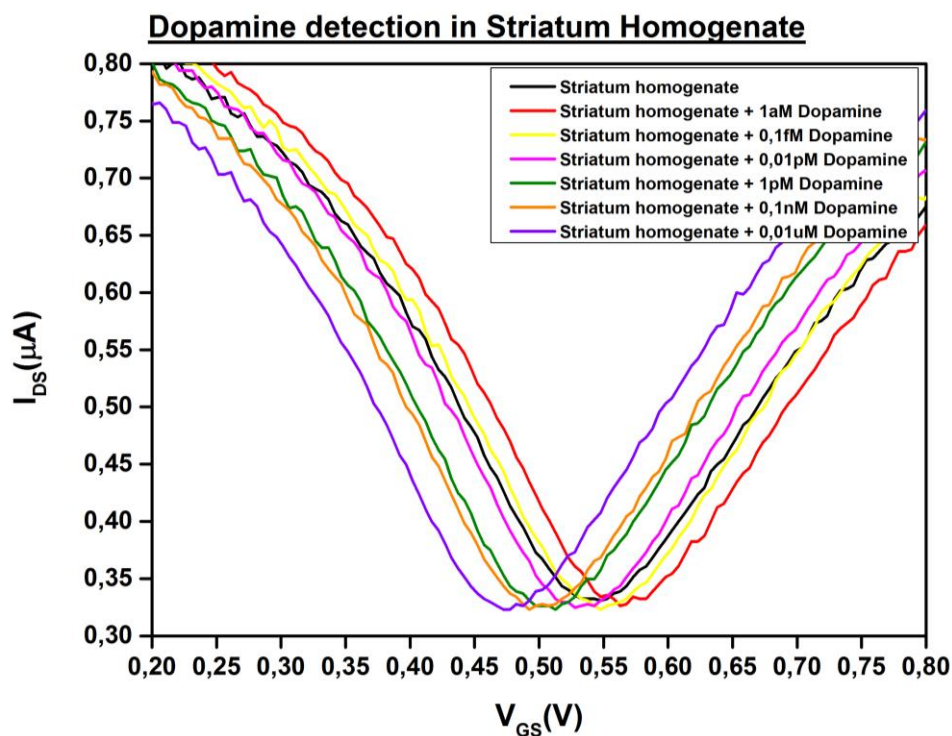


Figure 56. Graphene transfer curve for dopamine detection in striatum homogenate with measurements on striatum homogenate without dopamine.

7.3.1 Striatum homogenate control experiments

To validate that we were detecting dopamine and not other non-target molecules present in the brain tissue or that our measurements were being affected by the process itself, we performed two additional control experiments.

To control for the measurement and cleaning processes as well as for the incubation with multiple media (striatum homogenate, PBS 1x and striatum homogenate again) in a sequential fashion, we replicated the protocol described above but incubated striatum homogenate samples from a reserpine-treated animal without dopamine added only. Fresh 10 μ L samples of striatum homogenate were incubated for 20 minutes and measured as described for the experiments above. After the measurements, a new sample of striatum homogenate was incubated again. This process was repeated 4 times.

Such measurements can be found on figure 57, where we see measurements stabilization after 40 minutes. The three types of measurements have similar behavior overtime but symmetric to the baseline, meaning that there was no dopamine detected, otherwise all measurements would have the same negative linear shift trend.

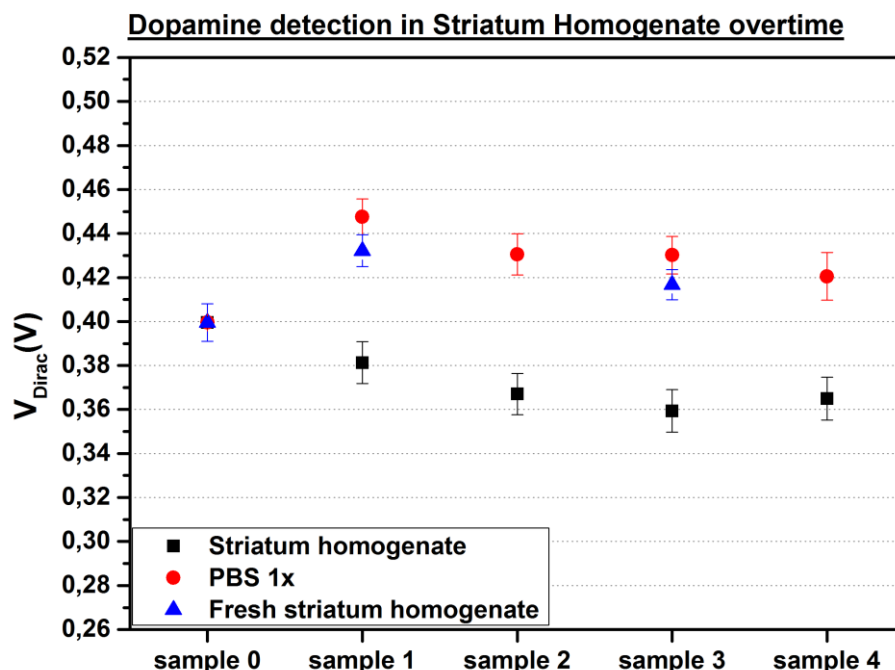


Figure 57. Overtime measurements of reserpine-injected striatum homogenate.

In a second control experiment, we assessed the effect of measuring the same striatum homogenate sample over a long period of time in order to determine how long dopamine would take to bind to the aptamers. A 10 μ L striatum homogenate sample from a control animal,

obtained as described for reserpine-treated animals, was incubated and measured on the same chip for 210 minutes in a humidity-controlled chamber to avoid evaporation and precipitation effects.

This experiment's data can be found in figure 58. At 30 minute mark, which corresponds to the first measurement taken, we observed a -30 mV shift of the Dirac point in respect to the baseline. At the 60 minute mark, an additional shift of -10 mV (-40 mV in respect to the baseline) was observed. The Dirac point then remained unchanged for the following 150 minutes. Meaning that there is no more dopamine to bind to the aptamers and that the linear range of detection for a control striatum homogenate is 40 mV.

Based on these two experiments we conclude that dopamine binding to the aptamers was not time-dependent nor medium dependent (there is no continuous molecule deposition overtime).

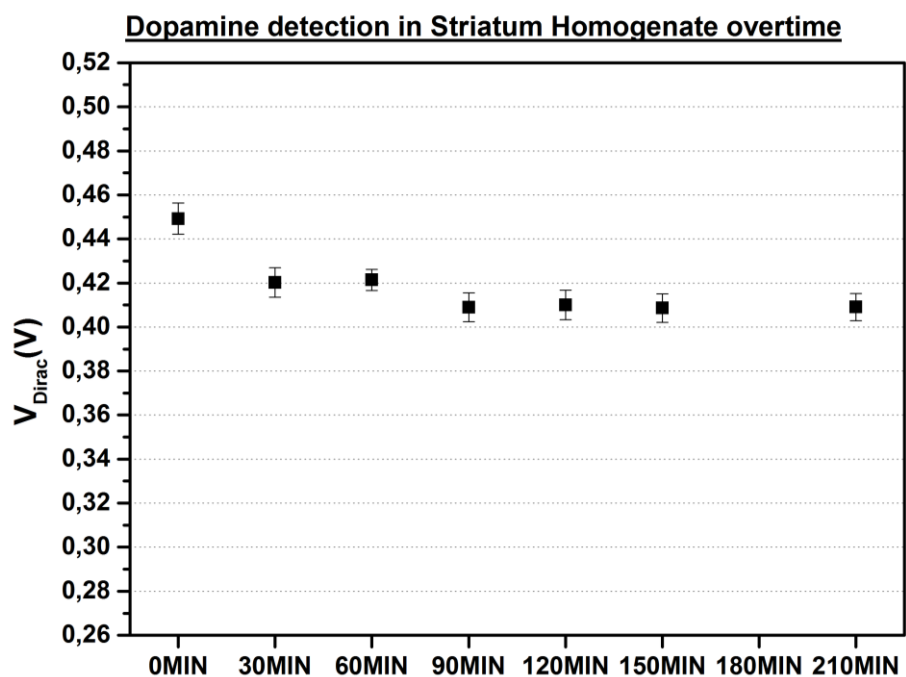


Figure 58. Overtime measurements of control striatum homogenate.

Dopamine detection with our sensors proved to be possible in a very complex medium that is the brain, obtaining a response of +45 mV in cerebrospinal fluid and detection on 0.1 fM for striatum homogenate.

These promising results allow us to consider detecting dopamine with our chips in an *in vivo* setting.

8. CONCLUSION

Detecting neurotransmitters for better understanding of how the human brain works is of upmost importance, and in the past few years, recent advances in this field were facilitated with the help of novel biosensors.

To current date, dopamine, a neurotransmitter with many important roles in the central nervous system, has been hardly detected below femtomolar concentrations. Not only some methods oxidize dopamine, but also use labelling agents, making detection impossible in a *in vivo* scenario. These and many other drawbacks of dopamine detection methods have been hindering the understanding of how dopamine works inside the brain and therefore, dopamine-related diseases.

In this work, we combined the excellent properties of a 2D material, graphene, with a small, robust, and highly adaptive probe, a DNA aptamer. The biggest benefit that graphene introduces to the biosensing field, is the ability to reach limits of detection never reported before due to its one atom thickness. Thus, sparse events occurring on functionalized graphene can be felt and measured for low voltages and currents.

Accordingly, label free highly sensitive and highly selective dopamine detection with electrolyte-gated graphene field-effect transistors was demonstrated in this work. It was the first time that our transistors had been functionalized with aptamers and our passivation method success was demonstrated based on the Dirac point voltage and x-ray photoelectron spectroscopy experiments.

The detection method based on local gating of the electrical double layers allowed several electrolyte solutions to be studied. In phosphate buffered saline electrolyte, which is the common buffer solution used in biological experiments, we obtained a positive Dirac point shift for all dopamine concentrations and the limit of detection, 1 attomolar. This is the lowest LOD ever reported for dopamine detection. The linear range of detection was 40 mV and surface saturation occurred at 1 pM.

Then, we moved on to a more complex electrolyte solution that could closely mimic the brain's medium, the artificial cerebrospinal fluid. In aCSF we presented a divalent cation aptamer dependence that altered and improved dopamine detection, increasing the transistors' linear range of detection from 40 mV to 140 mV and surface saturation occurred two concentrations higher, at 0.1 nM. The limit of detection was one concentration higher than in PBS, at 10 aM.

The selectivity of our sensors was also tested. The aptamer bonded specifically to dopamine only, giving barely any response to dopamine analogues and interfering agents like L-Dopa, L-Tyrosine, Homovanillic acid and Ascorbic acid.

With this state-of-the-art contender limit of detection for *in vitro* experiments, we transitioned to more complex electrolytes.

Dopamine detection was thus studied in *ex vivo* setting, where cerebrospinal fluid and striatum homogenate from mice were extracted and measured in our chips. In order to prove that our sensors' Dirac point shift was caused by dopamine only, we compared the measurements from control mice to reserpine-injected mice, the latter an acute animal model of dopamine depletion. In cerebrospinal fluid from control mice, our sensors gave a distinct response of around 45 mV more when compared to reserpine-injected mice. In striatum homogenate from dopamine depleted animals spiked with dopamine, the transistors gave a linear response for various dopamine concentrations with a linear range of 70 mV and the limit of 0.1 fM.

The linear range for PBS 1x was 40 mV, for aCSF was 140 mV and for striatum homogenate was 70 mV. Our aptamer conformational change is enhanced by divalent cations, which explains why the response is bigger in aCSF and striatum homogenate than on PBS 1x (which has only monovalent cations), despite having slightly lower limits of detection.

In conclusion, our graphene functionalized transistors are positioned in a state-of-the-art dopamine detection level and can be thoroughly improved with different graphene passivation methods to avoid non-specific binding effects, increase limits of detection and make it possible to progress into *in vivo* experiments. A possible future direction would be implementation of our graphene sensors in brains for better understanding of dopamine release through synaptic neurons and its acting mechanism inside the blood-brain-barrier. To do so, flexible graphene probes must be developed.

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SUPPLEMENT 1 – NANOPT2021 CONFERENCE’S ABSTRACT

This work’s results up to chapter 6 were presented in NanoPt2021 conference held on 16/09/2021 in speaking presentation format. The abstract was the following:

ATTOMOLAR LABEL-FREE DOPAMINE DETECTION WITH APTAMER FUNCTIONALIZED GRAPHENE FIELD-EFFECT TRANSISTORS

MAFALDA ABRANTES^{1,2,3,4}

P. D. Cabral^{1,4}, T. Domingues^{1,4}, J. Borme¹, L. Jacinto^{2,3}, P. Alpuim^{1,4}

¹*International Iberian Nanotechnology Laboratory, 4715-330, Braga, Portugal*

²*Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, 4710-057, Braga, Portugal*

³*ICVS/3B’s—PT Government Associate Laboratory, 4710-057 Braga/Guimarães, Portugal*

⁴*Department of Physics, University of Minho, 4710-057, Braga, Portugal*

mafalda.abrantes@inl.int

Dopamine is a neurotransmitter with critical roles in the human brain and body, and its dysfunction underlies brain disorders such as Parkinson’s Disease and schizophrenia. However, current sensors to detect dopamine in the brain lack relevant selectivity or sensitivity hindering the development of reliable diagnostics and the potentiation of earlier and more efficient treatment. Our previous work reported electrolyte-gate graphene transistors for DNA detection down to the attomolar level [1] using DNA functionalization and protein detection on a picomolar range [3] using antibody functionalization. In this work, we adapted this technology to detect dopamine by using a dopamine-specific aptamer for high selectivity and specificity [2]. The aptamer was immobilized onto a graphene field-effect transistor’s channel by a previously reported functionalization process with a pyrene derivate linker [3]. The sensor signal is the charge neutrality point, V_{Dirac} , in the transistor transfer curve.

Dopamine detection experiments were performed using phosphate buffered-saline solution (PBS 1x) and artificial cerebrospinal fluid (aCSF) as the electrolyte gate solution. Dopamine was diluted in these solutions from 0.1 pM to 1 aM. Figure 1 (left) shows the linear transistor response to dopamine, diluted in PBS 1x, from attomolar level to 0.01 picomolar concentration followed by

saturation at 0.1 picomolar, with 10mV/dec sensitivity. Figure 1 (right) shows selective dopamine detection compared with transistor's response to L-Dopa, L-Tyrosine, Homovanillic acid and, L-Ascorbic acid.

These results show that the combination of graphene field-effect transistors with a dopamine-specific aptamer allows highly selective and sensitive dopamine detection. The measured detection limit is the lowest ever reported for dopamine. These promising results allow us to consider further experiments in an *in vivo* setting.

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FIGURES

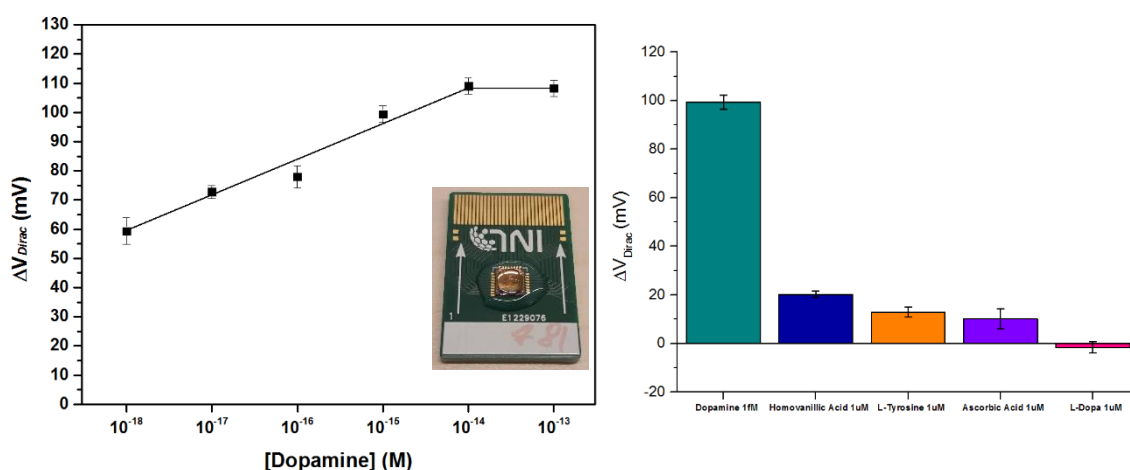


Figure 1: Calibration curve obtained measuring the Dirac point of 20 graphene EGFETs by applying a V_{DS} of 1mV and sweeping V_{GS} from 0V to 1V (left); a graphene EGFET chip is shown in the inset, and the sensors' response compared to non-specific targets, being all diluted in PBS 1x (right).

SUPPLEMENT 2 – SPN2021 CONFERENCE’S ABSTRACT

This work’s results up to chapter 7 were presented in SPN2021 conference held on 02/12/2021 in poster presentation format. The abstract was the following:

DOPAMINE DETECTION USING GRAPHENE APTASENSORS

Mafalda Abrantes^{1,2,3,4}, Diana Rodrigues^{2,3}, Telma Domingues¹, Patrícia Monteiro^{2,3}, Jérôme Borme¹, Pedro Alpuim^{1,4}, Luís Jacinto^{2,3}

¹International Iberian Nanotechnology Laboratory, 4715-330, Braga, Portugal

²Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, 4710-057, Braga, Portugal

³ICVS/3B’s—PT Government Associate Laboratory, 4710-057 Braga/Guimarães, Portugal

⁴Department of Physics, University of Minho, 4710-057, Braga, Portugal

Dopamine is a monoamine neurotransmitter with critical roles in the human brain and body, and its dysfunction underlies brain disorders such as Parkinson’s Disease and schizophrenia. However, current sensors to detect dopamine in the brain typically lack relevant selectivity or sensitivity, hindering the development of reliable diagnostics and potentiating earlier and more efficient treatment. In this work, we developed a highly sensitive and selective label-free sensor for dopamine detection based on graphene field-effect transistors functionalized with a dopamine-specific DNA aptamer.

The novel graphene aptasensor was initially validated *in vitro* with dopamine diluted in phosphate-buffered-saline solution (PBS) and artificial cerebrospinal fluid (aCSF) solutions. Our aptasensors achieved a detection limit as low as 1 aM, the lowest ever reported for dopamine, and a linear response range up to 1 pM. Additionally, our sensors proved to be highly selective for dopamine when compared with observed negligible responses to dopamine precursors such as L-Dopa, L-Tyrosine, and physiological electroactive interferents such as ascorbic acid.

Ex vivo validation experiments were also conducted in CSF and striatum homogenate samples from control and reserpine-treated animals, the latter a model of acute monoamine depletion. Our graphene aptasensors were able to detect dopamine added to striatal homogenates of dopamine-

depleted animals down to attomolar level, and differences in dopamine concentration in CSF samples from control and dopamine-depleted animals.

The measured detection limits are the lowest ever reported for dopamine both *in vitro* and in biological samples which allows us to consider further experiments in an *in vivo* setting.

SUPPLEMENT 3 – DOPAMINE DETECTION IN BLOOD

A substantial amount of dopamine circulates in the bloodstream, but in humans, over 95% of the dopamine in the plasma is in the form of dopamine sulphate. Since dopamine does not cross the blood-brain barrier, its synthesis and functions in peripheral areas are independent of its synthesis and functions in the brain. Therefore, dopamine detection outside the brain is not of upmost interest.

Blood samples from mice were measured in three different chips. On the first chip, chip 694, we incubated a blood centrifuged sample from a reserpine-injected animal and measured a +25 mV Dirac point shift.

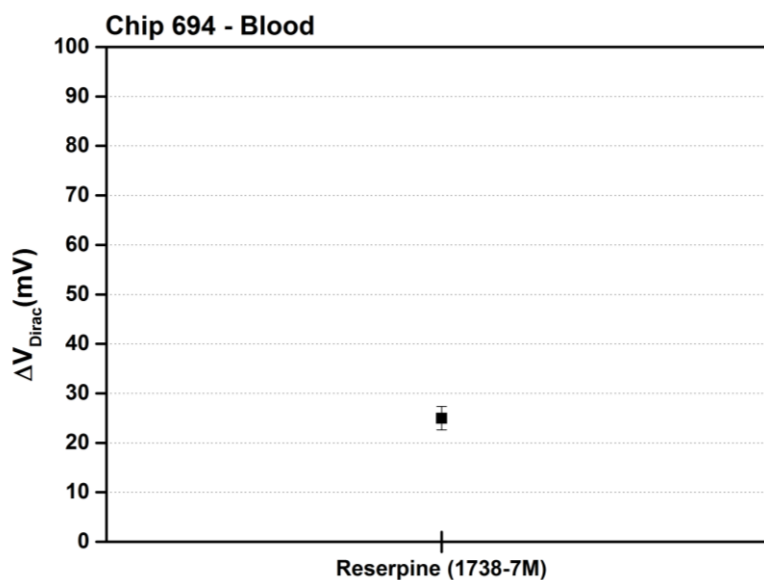


Figure A. Blood's dopamine detection for chip 694.

On the second chip, number 762, blood from a control animal gave a +30 mV Dirac point shift.



Figure B. Blood's dopamine detection for chip 762.

And for the last chip, we incubated two different blood samples, a reserpine-induced animal and control animal, giving +37 mV and +55 mV Dirac point shift respectively.

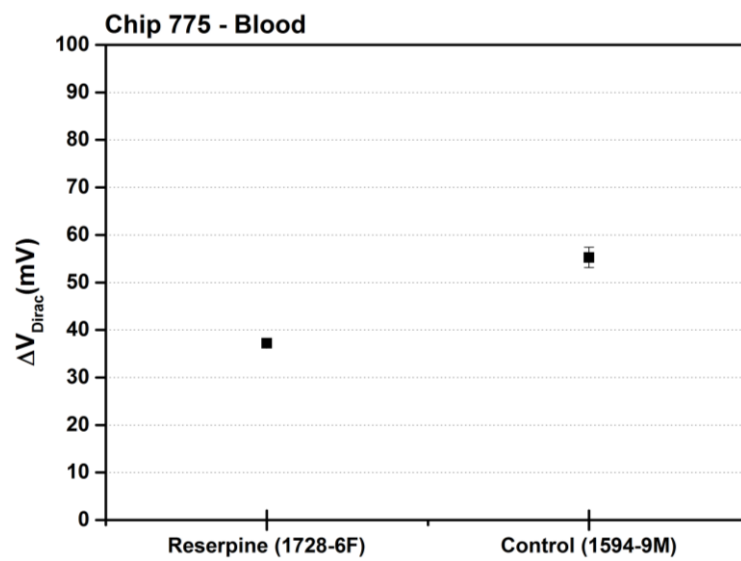


Figure C. Blood's dopamine detection for chip 775.

Since the chip with reserpine sample and the chip with control sample gave similar response and the difference between both steps on the same chip lacked a considerable distinction. We think that non-specific binds might be happening, therefore our sensors don't have a functionalization method for dopamine detection appropriate for a blood electrolyte.