
Imperial College London

Graphene field effect transistor biosensor platform
for detection of disease biomarkers in physiological
solutions

Tianyi Yin

Submitted for the degree of Doctor of Philosophy (Ph.D.)

Department of Materials

Imperial College London

2024

Abstract

Electrical biosensors based on graphene field-effect transistors (GFETs) have shown great potential for rapid and highly sensitive detection of a wide range of biomolecules. Biosensors based on GFETs have the potential to enable the development of point-of-care diagnostic tools for early-stage disease detection. They are biocompatible, easy to functionalize, scalable for mass production, and can be incorporated into portable devices for point-of-care diagnostics. However, sensitivity and selectivity are paramount for biomolecule detection in complex physiological solutions. Issues with reproducibility and uniformity of graphene sensors, but also with Debye screening and unwanted detection of non-specific species, have prevented the wider clinical use of graphene technology. This thesis attempts to address the critical challenges related to the performance limitations of GFETs in clinical settings, including sensitivity and selectivity. This thesis introduces strategies to enhance the performance and increase the accuracy of GFETs biosensors detection of disease biomarkers in complex physiological solutions.

Declaration

Originality declaration

I, the author, declare that the work presented in this thesis is my own work, from my own research. All contributions and assistance received have been properly acknowledged. Any other work used in the writing of this thesis is appropriately referenced.

Copyright declaration

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial 4.0 International Licence (CC BY-NC).

Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Acknowledgements

I would like to express my sincere gratitude to the following individuals and organizations for their invaluable support and contributions to the completion of this thesis and my PhD study.

Firstly, I would like to thank my first main supervisor, Prof. Norbert Klein. Regrettably, Prof. Norbert Klein passed away over the summer in 2023. His guidance and mentorship were throughout my academic journey, from undergraduate studies to my PhD. I feel so lucky to know Norbert and to be in his group. Norbert not only shared many scientific knowledge with me but also shared so much experience and thoughts about life and family with me. He guided me in developing my research skills, improving my writing skills, encouraging me and giving me opportunities for presentations, giving me advice for my future career. I learnt so much from him. Part of my achievement today is dedicated to him.

I am also grateful to our postdoctoral colleague, Dr. Sami Ramadan, whose constant support, and encouragement have greatly enriched my PhD experience. His generosity in sharing knowledge and expertise gave me many inspirations for my research and saved me a lot of time in making mistakes. I am really lucky to know him and to learn from his passion for science. I would not have achieved the results outlined in this thesis without his help and suggestions.

I extend my heartfelt appreciation to my second main supervisor, Prof. Neil Alford, for his constant encouragement and genuine care for my well-being. His warmth and support have provided me with the strength and motivation to finish my PhD.

I would also like to thank my brilliant research colleagues. Special thanks to Dr. Lizhou Xu for his ongoing support and guidance, particularly in the areas of exosomes and academic writing. His insights and visions have been invaluable. Special thanks to Dr. Bruno Gil Rosa, with whom I shared countless hours conducting the GFET measurements and clinical testing. He also helped in developing the portable electronic readout systems. I am very grateful to Dr. David Gaboriau for his support in helping with confocal fluorescent imaging; to Nabeel Merali, Dr. Adam Frampton, and Prof. Long R Jiao, our clinical partners, for their support in providing clinical samples for my research; to Xi Xu for her invaluable assistance and discussions on DFT simulation; to Dr. Maria S. Sokolikova for her assistance with Immunogold TEM; to Ben Chan

for helping with the 3D printing, to Ahmed, Sarah, and Jiapeng for their assistance in the laboratory. You have greatly facilitated the progress of my research. Every day has been enjoyable with all of you. I also wish to thank all my friends. I wish to acknowledge Dr. Elías Torres and Graphenea for supporting the commercially manufactured GFET devices for the clinical testing.

I also wish to thank my boyfriend, Zhiping Huang, for his unwavering emotional support, encouragement and advice. He gives me suggestions in my lab work, particularly in helping me with the design of 3D printing and PCA analysis. I would not have gone this far without you. You played a pivotal role in supporting me and encouraging me.

I owe deepest gratitude to my family, thank you for your support in the past 24 years. To my parents, thank you for allowing me to pursue the PhD and continuous support. I am thankful for the trust you put in me. You are part of this achievement. Without your encouragement and support, I'm sure I wouldn't have made it to where I am today.

Publications and Conferences

Journal publications:

Tianyi Yin et.al. “Progress in graphene field-effect biosensors for the detection of disease biomarkers in physiological solutions” in Progress

Tianyi et al. “Control of antibody orientation on graphene using porphyrin linker molecules for high-performance graphene-based immuno-biosensors,” Submitted.

“Yin, T., Xu, L., Bruno Gil Rosa, Nabeel Merali, Sokolikova, M.S., Gaboriau, D., Liu, D., Nizamuddin, A., Alodan, S., Chen, M., Oihana Txoperena, María Arrastua, Juan Manuel Gómez, Nerea Ontoso, EliceGUI, M., Torres, E., Li, D., Mattevi, C., Frampton, A.E. and Jiao, L.R. (2023). Graphene Sensor Arrays for Rapid and Accurate Detection of Pancreatic Cancer Exosomes in Patients’ Blood Plasma Samples. *ACS Nano*, 17(15), pp.14619–14631. doi:<https://doi.org/10.1021/acsnano.3c01812>.”

“Ramadan, S., Lobo, R., Zhang, Y., Xu, L., Shaforost, O., Kwong Hong Tsang, D., Feng, J., Yin, T., Qiao, M., Rajeshirke, A., Jiao, L.R., Petrov, P.K., Dunlop, I.E., Titirici, M.-M., Klein, N., 2021, “Carbon-Dot-Enhanced Graphene Field-Effect Transistors for Ultrasensitive Detection of Exosomes”, *ACS Appl. Mater. Interfaces* 13, 7854–7864”

“Xu, L., Ramadan, S., Rosa, B.G., Zhang, Y., Yin, T., Torres, E., Shaforost, O., Panagiotopoulos, A., Li, B., Kerherve, G., Kim, D.K., Mattevi, C., Jiao, L., Petrov, P., Klein, N., 2022, “On-chip integrated graphene aptasensor with portable readout for fast and label-free COVID-19 detection in virus transport medium”. *Sens. Diagn.* 10.1039.D2SD00076H”

Conference presentations and posters:

-
- Biosensors 2023 – 33rd Anniversary World Congress on Biosensors – Busan, Korea: 5-8 June 2023 (Oral Presentation)
Graphene sensor arrays for rapid and accurate detection of pancreatic cancer exosomes in patient's blood plasma samples
Tianyi Yin, Lizhou Xu, Bruno Gil Rosa, Nabeel Merali, Maria Sokolikova, David C. A. Gaboriau, Daniel Liu, Ahmad Nizamuddin Bin Muhammad Mustafa, Sarah Alodan, Elias Torres, Cecilia Mattevi, Adam E. Frampton, Long R. Jiao, Sami Ramadan, Norbert Klein
 - Biosensors 2023 – 33rd Anniversary World Congress on Biosensors – Busan, Korea: 5-8 June 2023 (Poster Presentation)
Comprehensive investigation of antibody orientation on graphene surface
Tianyi Yin, Sami Ramadan, Xi Xu, Zhiping Huang, Bruno Gil Rosa, Stephen Skinner, Norbert Klein
 - Graphene 2022 – 12th edition of Graphene Conference – Aachen, Germany: 5-8 July 2022 (Poster Presentation)
On-Chip Graphene Field-Effect Biosensors for the Detection of Glial Fibrillary Acidic Protein in Patient Plasma
Tianyi Yin, Lizhou Xu, Sami Ramadan, Oluwatomi E. Akingbade, Yuanzhou Zhang, Sarah Alodan, David J. Sharp, Bing Li, and Norbert Klein
 - Institute of Chemical Biology and Network for Sensor Technologies Biomimetics Symposium – London, United Kingdom: 22 June 2022 (Oral Presentation)
On chip integrated Graphene Field Effect Transistor sensor array for fast detection of neurodegenerative disease biomarkers and cancer-derived exosomes in clinical patients
Tianyi Yin, Lizhou Xu, Sami Ramadan, Oluwatomi E. Akingbade, Yuanzhou Zhang, Sarah Alodan, David J. Sharp, Bing Li, Adam Frampton, Long R Jiao, Norbert Klein
-

Table of Contents

Abstract.....	2
Declaration	3
Acknowledgements.....	4
Publications and Conferences.....	6
List of Tables.....	31
1 Aims and objective	32
Thesis Outline	33
2 Biosensing using graphene opportunities and challenges	37
2.1 Introduction to graphene and graphene field-effect transistors	40
2.1.1 Electronic properties of graphene	40
2.1.2 Graphene growth and fabrication	40
2.1.3 Graphene field effect transistor biosensor basic principle	43
2.2 GFET biosensors.....	44
2.2.1 Sensing mechanisms.....	44
2.2.2 Biofunctionalisation of graphene field effect transistors	46
2.2.3 Selection of bioreceptor molecules	56
2.3 Performance metrics and limitations in graphene field effect transistors	57
2.3.1 Challenges in Real Clinical Physiological Solutions	57
2.3.2 Strategies of sensing in physiological solutions	60
2.3.3 Overcoming the Debye screening effect	61
2.3.4 Strategies to overcome non-specific bindings.....	72
2.3.5 Reducing device noise for improved sensitivity	74
2.4 Applications of GFET biosensor in clinical studies	76

2.5	Summary	93
3	<i>Materials and Methods</i>	95
3.1	Graphene Transfer	95
3.2	Characterisation Methods.....	97
3.2.1	Optical Microscopy	97
3.2.2	Atomic Force Microscopy (AFM)	98
3.2.3	Fluorescent Microscopy.....	100
3.2.4	Scanning Electron Microscopy (SEM)	103
3.2.5	X-ray photoelectron spectroscopy (XPS)	104
3.2.6	Raman Spectroscopy	106
3.2.7	Electrochemical Impedance Spectroscopy (EIS)	110
3.2.8	Contact Angle Measurements	110
3.2.9	Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS).....	110
3.3	Fabrication of graphene field effect transistor sensor array	112
3.3.1	Cleaning and graphene modification.....	112
3.3.2	Graphene patterning and etching.	113
3.3.3	Contact opening and metallisation.....	113
3.3.4	Device passivation.	113
3.4	Preparation of biological fluids	114
3.4.1	Phosphate Buffered Saline (PBS) Solutions	114
3.4.2	Bovine Serum Albumin (BSA).....	115
3.4.3	Exosomes.....	115
3.4.4	Clinical samples.....	115
3.5	Functionalization of GFET biosensor.....	116

3.5.1	TCP Method	116
3.5.2	PBASE Method	117
3.5.3	Antibody Conjugation	117
3.6	Device Testing.....	118
3.6.1	Portable readout electronic system	118
3.6.2	Electrical measurements	119
3.6.3	Electrical Data Analysis	120
3.6.4	Microfluidic Channel Fabrication and Integration	123
3.6.5	Clinical testing protocol	126
3.7	Exosomes characterisation techniques.....	127
3.7.1	Nano-Flow Cytometry.....	127
3.7.2	Exosome isolation from human plasma samples	128
3.7.3	Transmission Electron Microscopy (TEM) and Immunogold labelling.....	129
3.7.4	Western blot analyses	131
3.7.5	Dynamic Light Scattering (DLS)	132
3.8	Density functional theory (DFT) Simulation	132
3.9	Statistical analysis.....	133
4	<i>Probing the Orientation of Antibodies on graphene biosensor</i>	<i>134</i>
4.1	Key Novelty and Achievements.....	134
4.2	Overview	135
4.3	Characterisation of graphene functionalized with TCP and PBASE linkers.....	136
4.3.1	Raman Spectroscopy	137
4.3.2	AFM	140
4.3.3	XPS	141

4.3.4	Water contact angle (WCA) measurements	143
4.4	Characterisation of antibody orientation on graphene surface with TOF-SIMS	145
4.4.1	Antibodies.....	146
4.4.2	Principal Component Analysis (PCA).....	147
4.4.3	Sample preparation and graphene functionalisation for TOF-SIMS measurement 149	
4.4.4	TOF-SIMS and PCA Analysis	149
4.4.5	Discussion	154
4.5	DFT simulation.....	159
4.6	Summary	168
5	<i>Role of linker molecules in performance of graphene biosensors</i>	<i>170</i>
5.1	Key Novelty and Achievements.....	170
5.2	Overview	171
5.3	Exosome and their role in cancer detection.....	171
5.4	Optimisation of GFETs for the detection of exosomes	174
5.4.1	GFET sensor array	174
5.4.2	On-chip gold gate electrode	175
5.4.3	Differential measurement	179
5.5	Comparative performance of GFETs with PBASE and TCPP in buffer	181
5.5.1	Electrical performance.....	181
5.5.2	AFM results.....	186
5.5.3	Confocal microscope results.....	188
5.6	Comparative performance for the detection of GPC1 using GFETs with PBASE and TCPP with clinical samples	189
5.6.1	AFM	190

5.6.2	Electrical performance.....	191
5.7	Summary	193
6	<i>Clinical detection of PDAC cancer exosome in patients</i>	<i>195</i>
6.1	Key Novelty and Achievements.....	195
6.2	Overview	197
6.3	Fabrication and characterization of GFET biosensor	199
6.3.2	Raman spectroscopy measurements.....	202
6.3.3	X-ray photoelectron spectroscopy (XPS) measurements	203
6.4	Clinical Cohorts	203
6.5	Selection of antibody for PDAC cancer exosome detection.....	205
6.6	Design and Optimization of the performance of GFET sensor array	206
6.7	Clinical detection of PDAC cancer patient samples with GFET.....	218
6.8	Validation of GFET response to GPC-1 exosome concentration.....	223
6.8.1	Nano Flow Cytometry	223
6.8.2	Surface characterisation techniques	226
6.8.3	Exosome isolation and characterisation of isolated exosomes from plasma samples.....	231
6.8.4	Discussion	235
6.9	Analytical modeling of GFETs response to GPC-1 exosome	236
6.10	Assessment of the GFET response versus the stage of cancer	240
6.11	Conclusion	241
7	<i>Conclusions</i>	<i>243</i>
8	<i>Outlook</i>	<i>246</i>
8.1	Performance enhancement for GFET sensing in physiological solutions	246
8.2	Multiplexed detection using GFETs biosensors.....	248

<i>References</i>	<i>250</i>
<i>Appendices</i>	<i>277</i>

List of Figures

Figure 2-1 A) Hexagonal lattice of graphene, two atoms (A and B) per unit cell. [27] B) Electronic structure of graphene: Energy bands near the Fermi level in graphene. The conduction and valence bands cross at the points K and K'. [28] C) Energy band of graphene, the conduction and valence bands touching at the Dirac point. The position of the Fermi level determines the nature of the doping and the transport carrier. [27]	40
Figure 2-2 A) Schematic of the CVD system.[16] B) Schematic diagram of the process of chemical vapor deposition procedure for graphene synthesis with Cu.[37]	42
Figure 2-3 Schematic diagram of A) a conventional FET device, B) a back gated GFET device, C) a GFET device, D) a liquid gated GFET device. [47] E) IV characteristic diagram of graphene's unique ambipolar effect with two types of charge carriers in graphene. The field effect can modulate the holes (red colour, in left) to electrons (grey colour, in right). The charge-neutrality point V_{CNP} is located at the transition between the hole and electron regime, where the current is at its minimum. F) Schematic diagram of the modulation of the Fermi level position as the function of doping, with n-type doping, pristine and p-type doping graphene.[48]	44
Figure 2-4 Graphene field-effect-transistor as a versatile platform for biomarkers detection. GFETs biosensor structure and its typical transfer characteristics. The binding of charged molecules on the graphene surface leads to a change in the Fermi level with the respect of Dirac point. Negative charges p-dope graphene lead to a right shift in the transfer curve, while positive charges n-dope graphene leads to a left shift.	45
Figure 2-5 Schematic representation of chemical functionalization of graphene surface for various range of biomarkers.....	46
Figure 2-6 Schematic of the structure of a diazonium salt and process of covalent attachment of a diazonium salt to the graphene sheet surface.	48
Figure 2-7 Schematic illustration for the process of covalent grafting of aryl diazonium radicals on graphene leading to oligomer formation.	49
Figure 2-8 Schematic demonstration of maximum theoretical coverage of covalently attached diazonium salt group.	50

Figure 2-9 Schematic diagram of the structure of (a) PBASE (b) TCPP.....	52
Figure 2-10 Schematic diagram of NHS ester tripod linker, with three pyrene "feet" for interaction with graphene sheet. [101].....	53
Figure 2-11 A schematic representation of different functionalization processes used in graphene biosensors and their effects on the structural and electronic properties of graphene.	54
Figure 2-12 Schematic diagram for shape of antibody.....	56
Figure 2-13 Schematic diagram of electric double layer formation and Debye screening at surface. [137].....	59
Figure 2-14 Schematics of nanostructured graphene and flat graphene.	61
Figure 2-15 Schematic demonstration of Debye volume on a concave and convex electrode surface, for comparison of the Debye volume to surface area ratio. The higher Debye volume indicated stronger charge screening effect (convex surface). [1]	62
Figure 2-16 Surface nanostructuring for enhancing GFETs biosensor performance. A) Carbon dot decorated graphene field effect transistor. Electrical detection of exosomes on ND-GFET (nondecorated GFET) and CDs-GFET sensors. Dirac voltage shift of GFETs for various concentrations of exosomes on ND-GFET and CDs-GFET sensors.[86] . B) Crumpled graphene Lateral image of the flat (top) and crumpled (bottom) graphene FET DNA sensors. DNA (red strand) is hybridized with probe DNA (black strand) on the graphene surface. I–V relationship of the flat (top) and crumpled (bottom) graphene FET sensors for DNA hybridization. DNA hybridization shifted the I–V curve according to the indicated concentrations. The crumpled graphene's I–V curve shift is significantly more significant than the flat device. [148]	63
Figure 2-17 Schematics of graphene field effect transistor biosensor with a layer of ion permeable layer. The comparison of GFET device response from time measurements for untreated and polymer coated graphene.	64
Figure 2-18 GFET device response for detection of PSA in 300 nM in 1X PBS with 1:2 PEG:ETA-, pure aptamer-, and 1:2 PEG:aptamer- functionalized GFET devices. [137]	65

Figure 2-19 Schematics of tailored bioreceptor molecules that can be immobilized on GFET biosensors.	66
Figure 2-20 Schematics of strategies for overcoming the Debye screening effect using GFET devices with tailored bioreceptor molecules and flexible receptor structures. A) The SARS-CoV-2 detection in universal transport media (UTM) (mimic swab sample) using the on-chip integrated graphene biosensor (Apt-GFET, aptasensor): the comparison results of the on-chip Apt-GFET biosensor (aptasensor) vs. the Ab-GFET biosensor for SARS-CoV-2 virus detection in UTM.[17] B) $\Delta I_{ds}/I_{ds0}$ versus t curves of g-FETs with TBA-functionalized MoLEMS in full serum (black) and upon the sequential addition of thrombin in $1\times$ TRB buffer (red) or full serum (blue). The green curve is measured in full serum by a g-FET with a TBA-functionalized 5 T aptamer (Apta-FET). The purple curve corresponds to a g-FET with TBA-functionalized MoLEMS after 15 days of incubation in full serum. [178]	68
Figure 2-21 Bioreceptor molecules with gene editing technology based on CRISPR-Cas systems.	69
Figure 2-22 Overcoming Debye screening by operating GFETs at radio frequency. A) Schematic of GFETs in heterodyne frequency mixing configuration. The interaction of the high frequency (10 MHz) with the biomolecule's dipoles can lead to a change in the mixing current that sensitive to the biomolecule's charges.[188] B) GFETs biosensor in reflectometry mode configuration at UHF. The measurements were performed at around 2 GHz. Dielectric modulated UHF signal was demonstrated for the detection of streptavidin and cardiomyocyte cell on graphene. The method shows a detection of the signal beyond Debye screening compared to conventional GFETs. In the figure, dielectric modulation signal is represented in red, while the GFETs signal represented in black. [187].....	71
Figure 2-23 Schematics of techniques for overcoming the non-specific bindings. Schematics of various techniques that can be applied, including the conventional methods with blocking agents or the use of surface treatment to react the unreacted reactive groups, blocking via the full coverage of bioreceptor molecules.....	72
Figure 2-24 Examples of strategies for overcoming the non-specific binding. A) The MoLEMS have high coverage of the surface of graphene, the rigid bases avoid non-specific	

adsorption.[178] B) Schematic illustration of technique for prevention of non-specific binding by a pristine graphene-based FET sensor and a COF _{TAPB-BMTTPA} /graphene-based FET sensor. The black and red circles show the processes at the sensing interfaces.[207]	74
Figure 3-1 Schematic diagram of the wet graphene transfer process.	97
Figure 3-2 Olympus System BX51 compound optical microscope. [224]	98
Figure 3-3 Atomic force microscopy. A) Asylum MFP-3D classic atomic force microscope. B) The primary components of an atomic force microscope, including a 3D piezoelectric scanner with Z Scanner Electronics and X-Y Scanner Electronics, a laser, cantilever, and photodetector.	100
Figure 3-4 Fluorescent microscope. A) A Leica SP8 point scanning microscope. B) The primary components of a fluorescent microscope.	101
Figure 3-5 Schematic of a basic XPS instrument set-up.	105
Figure 3-6 A) Schematic diagram of processes of an emission of a photoelectron resulting from x-ray incident on surface. B) Energy level diagram of the schematic diagram of the basic XPS equation.	106
Figure 3-7 Energy-level diagram of the elastic and inelastic scattering mechanisms and the states involved in Raman spectra.....	107
Figure 3-8 Raman spectrum of pristine (top) and defected (bottom) graphene, with main peaks labelled. Single layer graphene can be identified by the intensity ratio of the 2D-band to G-band.[229]	109
Figure 3-9 A schematic diagram of the basic components and the fundamental principles of ToF-SIMS analysis.	112
Figure 3-10 Schematic representation of the GFET fabrication steps.	114
Figure 3-11 Illustration of the testing device with electronic readout measurement unit. .	119
Figure 3-12 Characteristic transfer curves of a GFET, with key features illustrated.	121
Figure 3-13 Typical output curves of a GFET.	123

Figure 3-14 Schematic diagram of the step-by-step fabrication of steps for the microfluidic channel. A) Schematic process for the fabrication of PDMS ring. i) Negative mould with 2 parts are 3D printed with the desired PDMS ring size. ii) The negative mould was assembled. The PDMS mixture were mixed at the desired ratio and ultracentrifuged to remove bubbles. The prepared PDMS mixture was carefully poured into the mould and left for curing overnight. iii) The PDMS ring was then removed from the mould, trimmed, and cleaned for further use. B) Schematic diagram of the CAD designed 3D printed parts of the microfluidic channel. These printed parts and the PDMS ring were inserted under the microfluidic reservoir for the assembly of the microfluidic channel for GFET testing.	126
Figure 3-15 Schematic illustration of the flow cytometry analysis of EV samples.	128
Figure 3-16 Transmission Electron Microscopy (TEM) and Immunogold labelling. A) The primary components of a TEM. B) Schematic representation of immunogold labelling mechanisms.....	130
Figure 4-1 Schematic diagram of surface-immobilized antibodies with different possible orientations on graphene biosensors functionalized using PBASE and TCPP as linker molecules.	136
Figure 4-2 A) Raman spectra of bare graphene and PBASE-functionalized graphene. (B) Raman spectra of bare graphene and TCPP-functionalized graphene.	138
Figure 4-3 Raman mapping of graphene surfaces functionalized with PBSAE and TCPP before and after immobilisation with antibody. Raman mapping experiment was conducted over a $4 \times 4 \mu\text{m}^2$ area of graphene with PBASE and TCPP, both with and without antibodies.....	139
Figure 4-4 Raman spectra of graphene functionalized with TCPP and PBASE before and after immobilization with antibodies.	140
Figure 4-5 Atomic force microscopy (AFM) profile for characterization of: (A) PBASE-modified graphene, graphene surface after PBASE + antibody biofunctionalization; (B) TCPP-modified graphene, and graphene surface after TCPP + antibody biofunctionalization. AFM scale bars = $1 \mu\text{m}$	141

Figure 4-6 N1 s of X-ray photoelectron spectroscopy (XPS) spectra of graphene with different degrees of functionalization: (A) bare, with PBASE, and with PBASE + antibody respectively; (B) bare, with TCPP, and with TCPP + antibody respectively.....	142
Figure 4-7 C1 s of X-ray photoelectron spectroscopy (XPS) spectra of graphene with different degrees of functionalization: (A) bare, with PBASE, and with PBASE + antibody respectively; (B) bare, with TCPP, and with TCPP + antibody respectively.....	143
Figure 4-8 Images of WCA measurements of SiO ₂ /Si substrates with and without graphene and after surface functionalization with linker molecules. WCA measurements of A) SiO ₂ /Si, B) G/SiO ₂ /Si, C) PBASE/G/SiO ₂ /Si, D) TCPP/G/SiO ₂ /Si.....	144
Figure 4-9 Schematic diagram for SIMS with PCA for antibody orientation characterization. In SIMS, three different antibody fragments were chosen for the analysis of orientation. A well-studied IgG antibody with known amino acid sequence was chosen.	146
Figure 4-10 Architecture of an antibody.	147
Figure 4-11 Schematic diagram of main steps in principal component analysis (PCA).	149
Figure 4-12 Example positive ion TOF-SIMS spectra of adsorbed IgG, F(ab) ₂ , and Fc on PBASE-functionalized and TCPP- functionalized graphene.	153
Figure 4-13 Principal component analysis of F(ab') ₂ and Fc antibody fragments immobilized on functionalized graphene surfaces. Score plot of PC 1 vs. PC 2 for the developed PCA model for PBASE- and TCPP-functionalized graphene surfaces. A TOF-SIMS peak list of 36 amino acid mass fragments was utilized. The ellipses drawn around each of the groups represent the 95% confidence limit for that group on PC1 and PC2.	155
Figure 4-14 Loading plot for PC2 in the developed PCA model for the recorded TOF-SIMS. (Figure 4-13) Ion fragments of amino acids abundant in the Fc domain load in the positive direction, while fragments of amino acids with higher content in the F(ab') ₂ domain load in the negative direction. The loadings are ordered in increasing mass-to-charge ratio.	155
Figure 4-15 Principal component analysis of the antibody fragments (F(ab') ₂ and Fc) and IgG immobilized on functionalized graphene surfaces, showing PC 1 vs. PC 2 score plot for the developed PCA model for: (A) PBASE functionalized graphene surfaces; and (B) TCPP	

functionalized graphene surfaces. A TOF-SIMS peak list of 36 amino acid mass fragments was utilized. The ellipses drawn around each of the groups represent the 95% confidence limit for that group on PC1 and PC2.....	158
Figure 4-16 Configuration of PBASE0, with atoms coloured as follows: grey = C; white = H; red = O; blue = N.	159
Figure 4-17 Configurations of PBASE with different rotations of its alkyl chain. The corresponding energy of each specific configuration of PBASE is shown in bracket. Atoms are coloured as follows: grey = C; white = H; red = O; blue = N.	161
Figure 4-18 DFT simulation of optimized PBASE structures on the graphene substrate for configurations with different angles of rotation. The PBASE0 configuration is used as the initial configuration before the rotation of the bonds. The relative energy of the PBASE molecule is a function of its configuration with different degrees of the angle of rotation. Black lines correspond to the configuration energy of the PBASE molecule as a function of the rotation angle, and red lines correspond to the adsorption energy of the PBASE configuration with respect to binding on the graphene surface.	162
Figure 4-19 DFT simulation of optimized PBASE structures on the graphene substrate for configurations with different angles of rotation. The PBASE0 configuration is used as the initial configuration before rotation of the bonds, marked as the 0 angle. The relative energy of the PBASE molecule is a function of its configuration with different rotation angles. Black lines refer to the configuration energy of the PBASE molecule as a function of the rotation angle, red lines correspond to the adsorption energy of the PBASE configurations with respect to binding on the graphene surface. (A) Rotation 1 around C-O-N (PBASE 0, PBASE-A1-3 in Figure S6); (B) Rotation 2 around C-O-N (PBASE 0, PBASE-B1-3 in Figure S6). The rotation of the PBASE end group is demonstrated at the bottom of each plot. Atoms are coloured as follows: grey = C; white = H; red = O; blue = N.	163
Figure 4-20 DFT simulation of configurations of PBASE. Optimized structure of PBASE2 and its configuration upon adsorption onto graphene (grey: C; white: H; red: O; blue: N).	165
Figure 4-21 DFT calculation of charge density difference map when PBASE2 is adsorbed onto graphene. In the charge density of isosurfaces, yellow indicates regions gaining charge and	

blue indicates regions losing charge. Cyan and yellow indicate charge accumulation and depletion respectively.	165
Figure 4-22 Characteristic I–V curves of GFET showing changes in the Dirac point after surface functionalization with PBASE.....	166
Figure 4-23 Optimized geometry of TCPP molecule. Atoms are coloured as follows: grey = C; white = H; red: O; blue = N.	167
Figure 4-24 Optimized structure of TCPP, and its configuration upon adsorption onto graphene (grey: C; white: H; red: O; blue: N).	167
Figure 5-1 Schematic illustration of the PBASE-functionalized GFET and TCPP-functionalized GFET sensors. Schematic illustrations of the exosomes captured on PBASE-functionalized GFET and TCPP-functionalized graphene. The functionalization with TCPP enhances capture of the exosome and leading to higher device sensing signal.	171
Figure 5-2 Schematic diagram of the structure of an exosome, which is a cell-to-cell transit system with pleiotropic functions, it is consisted of phospholipid bilayer, multiple transmembrane proteins and other contents. [274]	172
Figure 5-3 Optical microscopy images of the GFET device A) before the passivation B) after the passivation. Images are taken at 20x magnification.	175
Figure 5-4 Validation of the performance of fabricated GFET biosensor with solution gate. Representative I_{DS} - V_{GS} curve of the GFET biosensor with A) Pt wire gate electrode, the tested GFET devices are with a graphene channel area of $20\ \mu\text{m} \times 10\ \mu\text{m}$, B) Ag/AgCl gate electrode, and C) on-chip integrated Au electrode. The response recorded with the Ag/AgCl gate electrode (B) and on-chip integrated Au electrode (C) are tested using the same GFET device with a graphene channel area of $100\ \mu\text{m} \times 100\ \mu\text{m}$. The drain voltage V_d for the electrical measurements are labelled in the plot. All measurements are performed using 0.001 x PBS.	176
Figure 5-5 Representative I_{DS} - V_{GS} curve of the GFET biosensor with the Pt wire gate electrode at 3 different devices. The drain voltage V_d for the electrical measurements are set as 0.1 V. All measurements are performed using 0.001 x PBS. The fabricated GFET devices are with a graphene channel area of $20\ \mu\text{m} \times 10\ \mu\text{m}$	177

Figure 5-6 Series of repeated transfer curve measurements of GFETs with on-chip integrated Au electrode in PBS. The data shows that the transfer curve become stable after the 5 th measurements.....	178
Figure 5-7 Monitoring of I–V curves over time on the same device. $V_{sd} = 20$ mV, indicating good stability of the data produced by each device over time.	179
Figure 5-8 GFET device shift of the control and sensing channels on three GFET chips tested for the same concentration of exosomes in buffer, illustrating the importance of control channel. The GFET device detection signal is calculated based on the difference between the GFET response from control and sensing channels. Error bars are determined by the standard deviation of multiple device measurements, $5 \leq n \leq 6$ for each reading.	180
Figure 5-9 Atomic force microscopy images of GPC-1 antibody functionalized graphene surface incubated with MCF-7 exosomes (model exosomes) in buffer, suggesting the expression of GPC-1 on the surface of MCF-7 exosomes.....	180
Figure 5-10 Calibration curve of the GFET biosensor for detection of various concentrations of spiked model exosomes in plasma. Error bars are determined by the standard deviation of multiple device measurements, $4 \leq n \leq 24$ for each reading. Differential response represents the device signal, which is the difference between sensing channel and control channel.	181
Figure 5-11 Characteristic I–V curves of GFET showing changes in the Dirac point after surface functionalization with PBASE and TCPP.....	182
Figure 5-12 Validation of TCPP-functionalized GFET biosensor for detection of exosomes with protocol using control and sensing channels. Detection of cancer exosomes in buffer using the GFET biosensor. Representative I_{DS} - V_{GS} curve of control channel and sensing channel on the GFET biosensor after exosome binding. A significant curve shift is seen in the sensing channel in comparison to the control channel, suggesting that the shift arises from exosome binding on the sensing channel.	183
Figure 5-13 Representative differential I_{DS} - V_{GS} curve of the TCPP functionalized GFET biosensors in PBS buffer with various concentrations of spiked exosomes.....	183

Figure 5-14 Validation of PBASE-functionalized GFET biosensor for detection of exosomes with protocol using control and sensing channels. Detection of cancer exosomes in buffer using the GFET biosensor. Representative I_{DS} - V_{GS} curve of control channel and sensing channel on the GFET biosensor after exosome binding. A significant curve shift is seen in the sensing channel in comparison to the control channel, suggesting that the shift arises from exosome binding on the sensing channel.	184
Figure 5-15 Representative differential I_{DS} - V_{GS} curve of the PBASE functionalized GFET biosensors in PBS buffer with various concentrations of spiked exosomes.	184
Figure 5-16 Calibration curves of the PBASE and TCPP functionalized biosensor for detection of different concentrations of spiked exosomes in PBS buffer. Error bars are determined by the standard deviation of multiple device measurements, $5 \leq n \leq 24$ for each reading. The baseline that represents LoD is defined by (response)PBS + 3*standard deviation = 3.6 mV ($n = 24$).	186
Figure 5-17 Atomic force microscopy (AFM) profile for characterization of exosome expression after 15 min incubation of 10^7 particles/ μ L exosomes on PBASE modified graphene with antibody biofunctionalization and TCPP modified graphene with antibody biofunctionalization. AFM scale bars = 1 μ m.	186
Figure 5-18 Exosome count on GFET sensors taken from an average of five spots on each of the functionalized graphene surfaces incubated with exosomes. (****P < 0.0001). AFM scale bars = 1 μ m. Data mean $\pm$ SD.	187
Figure 5-19 Histograms of height of exosomes as measured by AFM of PBASE and TCPP functionalized graphene with immobilized antibody after exosome incubation. The data were obtained on average from the analysis of around five $5 \times 5 \mu\text{m}^2$ AFM images.	188
Figure 5-20 PBASE and TCPP Functionalized graphene surface with immobilized antibodies incubated with spiked fluorescent exosomes in PBS buffer. Exosome signal is shown in grey scale, and the insert shows the same field of view with the detected exosomes after bioimage analysis in green to make it clearer. Confocal scale bars = 5 μ m.	188
Figure 5-21 Fluorescence confocal microscopy images of exosome count on TCPP and PBASE functionalized graphene, taken from an average of five different spots on the TCPP modified	

graphene and five different spots on the PBASE modified graphene. (For details of the method used to count exosomes, see “Materials and Methods”). (****P < 0.0001).	189
Figure 5-22 Characterization of surface functionalization of the GFET biosensor. (A) AFM profile of exosome expression on PBASE- and TCPP-functionalized graphene substrates immobilized with GPC-1 antibodies after 15 min incubation with PDAC patient plasma. AFM scale bars = 1 μ m.	191
Figure 5-23 Numbers of particles captured by GPC-1 antibodies immobilized on 25 μ m ² of PBASE- and TCPP-functionalized graphene surface incubation with healthy and PDAC patient plasma for 20 min (**P < 0.01, ***P < 0.001, ****P < 0.0001, n = 3). Data are mean $\pm$ SD.	191
Figure 5-24 Detection of cancer exosomes in PDAC patient plasma using the GFET biosensor. Representative I_{DS} - V_{GS} curve of (A) control channel and (B) sensing channel on the GFET biosensor after exosome binding. A significant curve shift is seen in the sensing channel in comparison to the control channel, suggesting that the shift arises from exosome binding on the sensing channel.	192
Figure 5-25 The measurement results for the GFET biosensors with 2 healthy plasma and 2 PDAC patients plasma illustrate the excellent sensitivity and selectivity of the TCPP-functionalized GFET biosensor (****P < 0.0001, n = 6). Data are mean $\pm$ standard deviation (SD).	193
Figure 6-1. Schematics of detection of PDAC exosomes using GFETs with portable electronics and real-time detection results. The total detection time from applying blood plasma on the GFETs to results is less than 45 min. The zoomed in area shows schematics of the functionalization of graphene with TCPP [317] and the GPC-1 antibody. The middle-right images are fluorescence images showing higher density of exosomes on the GFET surface for the PDAC patients’ sample than the healthy controls’. The top-right images are TEM images with immunogold labelling with GPC-1 to compare the GPC-1 proteins expressions on healthy and cancerous exosomes.	199
Figure 6-2 Typical output curve of the GFETs chips measured at room temperature.	200
Figure 6-3 Typical transfer curve of the GFET chips with liquid gating, where the resistance of the device is measured as a function of the gate voltage V. a) An external Ag/AgCl electrode	

is used with the PBS buffer solution. b) The on-chip Au electrode is used with the PBS buffer solution.....	200
Figure 6-4 Representative I_{DS} - V_{GS} curve of the bare GFET chips tested in the PBS buffer solution for 5 continuous sweep readings prior to the functionalization steps for quality control. ...	201
Figure 6-5 Example of the 5 continuous readings of the Dirac point of the GFET devices tested in the PBS buffer solution, illustrating low drift and stability of the GFET biosensor.....	201
Figure 6-6 Characterization of surface functionalization of the GFET biosensor. Raman spectra of bare graphene and TCPP-functionalized graphene.....	202
Figure 6-7 Characterization of surface functionalization of the GFET biosensor. C1 s and N1 s X-ray photoelectron spectroscopy (XPS) spectra of graphene with different degrees of functionalization: bare, with TCPP, and with TCPP + antibody respectively.	203
Figure 6-8 Selection and validation of antibodies for the clinical detection of pancreatic cancer cell-derived exosomes in patient plasma: A) AFM profile of exosome expression on 4 different antibodies functionalized on TCPP decorated graphene substrate (CD63, GPC-1, Prom1, and RhoB antibodies) after 15 min incubation with healthy plasma and PDAC patient plasma. AFM scale bars = 1 μ m. B) Numbers of particles captured by CD63, GPC-1, Prom1, and RhoB antibodies functionalized on 25 μ m ² of graphene surface after 20 min incubation with healthy plasma and PDAC patient plasma (**P < 0.01, ***P < 0.001, ****P < 0.0001, n = 4). Data are mean $\pm$ s.d.	206
Figure 6-9 Schematic flow diagram of the protocol implemented for the GFET testing with the exclusion and inclusion criteria applied.	208
Figure 6-10 A) Illustration of the layout of on-chip integrated GFET sensor, designed with 6 devices each on the left and right sides. These devices are separated by the central common-on-chip integrated Au electrode. B) Illustration of the optical image of on-chip integrated GFET devices.....	208
Figure 6-11 Illustration of the graphene chip with the 3D printed microfluidic reservoir, and PCB board-based portable electronics. Top view : left, side view : right.	209

Figure 6-12 Schematics of the on-chip integrated GFET biosensor platform with the antibody functionalized channel as the sensing channel and a channel without antibody functionalization as the control channel.	210
Figure 6-13 GFET device detection signal comparison between the five chips tested for the same concentration of exosomes in buffer, illustrating low device-to-device variation and high chip-to-chip reproducibility of the GFET biosensor. Error bars are determined by the standard deviation of multiple device measurements, $4 \leq n \leq 6$ for each reading.	211
Figure 6-14A) Comparison of device shift from sensing channel with different times of rinse. The decrease in device shift is due to the complex environment of blood plasma samples. The high salinity and sticky nature of blood plasma led to salt accumulation and left unwanted residues on the surface of GFET sensors B) Comparison of channel shift in control and sensing channels when testing with pure buffer and healthy plasma sample.....	212
Figure 6-15 Atomic force microscopy images of A) representative control channel of developed GFET sensor after incubation and testing with plasma sample. B) representative sensing channel of the developed GFET sensor after incubation and testing with plasma sample. C) Exosome count on GFET sensors taken from an average of five spots on each of the functionalized graphene surfaces incubated with plasma samples. Data are mean $\pm$ standard deviation (s.d.).....	214
Figure 6-16 Device exclusion criteria for GFET reproducibility and validity. Histogram of Dirac voltage for lab-tested GFET devices with Gaussian distribution curve. Device exclusion criteria were set based on 444 lab-tested GFET devices, where the Dirac voltage for lab-tested devices was $V_D = 0.71 \pm 0.24$ V.	215
Figure 6-17 Validation of GFET biosensor for detection of exosome with protocol of using control and sensing channel. Detection of cancer exosomes in buffer using the GFET biosensor. Representative I_{DS} - V_{GS} curve of the A) control channel and B) sensing channel on the GFET biosensor after exosome binding. Significant curve shift seen in the sensing channel in comparison to the control channel, suggesting that the shift arise from exosome binding on sensing channel. C) Representative I_{DS} - V_{GS} curve of the sensing channel of GFET biosensor in buffer with various concentrations of exosomes.	216

Figure 6-18 Calibration curve of the GFET biosensor for detection of various concentrations of model cancerous exosomes in buffer. Error bars are determined by the standard deviation of multiple device measurements, $4 \leq n \leq 24$ for each reading.	217
Figure 6-19 Measurement results for the GFET biosensor with healthy plasma and model cancer plasma, illustrating excellent selectivity of the GFET biosensor. (****P < 0.0001). Data are mean $\pm$ standard deviation (s.d.).....	218
Figure 6-20 A) Representative I_{DS} - V_{GS} curve of one of the control channels on the GFET biosensor before and after PDAC plasma incubation. The inset image shows the response from 6 control channels on the GFET biosensor. B) Representative I_{DS} - V_{GS} curve of one of the sensing channels on the GFET biosensor before and after PDAC plasma incubation, showing a significant curve shift in the sensing channel.	219
Figure 6-21 Device shift of the 6 control and 6 sensing channels on a representative clinically tested GFET device. The device shift arising from a plasma sample is calculated based on the difference between the GFET response from control and sensing channels.	220
Figure 6-22 Measurement results for the GFET biosensor from the clinical testing with plasma samples from 18 PDAC patients and 8 healthy controls, indicating a clear threshold detection signal line which suggests that the sensor is able to differentiate PDAC patients from healthy controls (****P < 0.0001). Data are mean $\pm$ standard deviation (s.d.).	221
Figure 6-23 Device exclusion criteria for GFET reproducibility and validity. Results from n = 281 clinically tested devices that satisfied the exclusion criteria are included in the study. Devices (n=25) with $V_D < 0.47 V$ or $V_D > 0.95 V$ were excluded in this study. Devices (n=12) that exhibited values of $R > 1700 \Omega$ or $R < 700 \Omega$ were also excluded.	222
Figure 6-24 Principal component analysis (PCA) plot of the relevant channel shift signal for PDAC and control group. PC1 captured the greatest variance of the data set, (99.08%). A clear separation line can be drawn on the PC1 of the PCA plot. This separation line can well separate the healthy control from the PDAC patient.....	223
Figure 6-25 Example size distribution of particles in blood plasma samples prior to ultracentrifugation.	224

Figure 6-26 Fluorescent intensity of the background for A) control measurement with blank PBS and B) sample measurement.....	225
Figure 6-27 Fluorescent intensity recorded for samples with different serial dilution factors. A) Sample diluted to 1 in 100 and B) 1 in 50.	225
Figure 6-28 Validation the detection of PDAC cancer exosomes in PDAC patient and healthy control plasma. Concentrations of GPC-1+ exosomes detected in 18 PDAC patient plasma samples and 8 healthy controls plasma samples by Nano Flow cytometry measurements. Comparison of numbers of PDAC and healthy exosomes captured on GFET biosensors. ...	226
Figure 6-29 Fluorescence confocal microscopy images of exosome on GFET sensors. A) Functionalized graphene surface with no exosome as control. B) Functionalized graphene surface incubated with healthy plasma sample (PS1). C) Functionalized graphene surface incubated with PDAC plasma sample (PS3). Exosome signal is shown in grey scale, and the insert shows the same field of view with the detected exosomes after bioimage analysis in green' to make it clearer. Confocal scale bars = 5 μ m.	227
Figure 6-30 Fluorescence confocal microscopy images of exosome count on GFET sensors taken from an average of three positions on the functionalized graphene surface incubated with PDAC plasma sample and three positions incubated with healthy plasma sample (****P < 0.0001).....	228
Figure 6-31 Atomic force microscopy images of functionalized graphene surface incubated with healthy plasma sample (PS1). G) AFM images of functionalized graphene surface incubated with PDAC plasma sample (PS3). AFM scale bars = 1 μ m.....	229
Figure 6-32 Exosome count on GFET sensors taken from an average of eight spots on each of the functionalized graphene surfaces incubated with healthy plasma samples (PS1 and PS2) and eight spots on incubated with PDAC plasma samples (PS3 and PS4). (**P < 0.01, **** P < 0.0001). Data are mean $\pm$ s.d.....	229
Figure 6-33 Comparison of exosomes captured on functionalized GFET sensors, scanning electron microscopy analysis of exosomes from healthy plasma samples: A) PS1 and B) PS2 and PDAC plasma samples. C) PS3 and D) PS4 captured on the functionalized graphene surface. SEM scale bars = 200 nm.....	230

Figure 6-34 Exosome counts on graphene incubated with different samples, taken from an average of four spots on each graphene surface.	230
Figure 6-35 Dynamic light scattering measurements of isolated exosomes from blood plasma samples. The DLS showed an average size of 76.5 nm, with the size of particles lies between 30 and 130 nm.....	231
Figure 6-36 Western blot analyses of exosomes isolated from 3 PDAC patients' plasma, with ALIX antibody, CD9 antibody, and GPC-1 antibody.	232
Figure 6-37 Western blot and IG-TEM validation of GPC-1 expression on PDAC cancerous and healthy exosomes. A) Western blot of GPC-1, CD9 and CD63 in exosomes extracted from healthy plasma and PDAC patient plasma.....	233
Figure 6-38 A) Low-magnification IG-TEM image of GPC-1 in PDAC cancer exosomes with immunogold labelling. The outlines of individual exosomes are highlighted by dashed yellow line for clarity. B) High-resolution IG-TEM image of GPC-1 on the surface of a PDAC cancer exosome with immunogold labelling. Inset shows the respective FFT pattern with the prominent ring corresponding to the (200) planes of Au. C) Low-magnification IG-TEM image of a healthy exosome with immunogold labelling. The yellow circles around individual exosomes are given for visual guidance. The arrows point to the individual Au particles on the surface of a healthy exosome. D) High-resolution IG-TEM image of GPC-1 on the surface of a healthy exosome with immunogold labelling.	234
Figure 6-39 A) Low-magnification IG-TEM image of GPC-1 in PDAC cancer exosomes with immunogold labelling. The outlines of individual exosomes are highlighted by dashed yellow line for clarity. B) Low-magnification IG-TEM image of a healthy exosome with immunogold labelling. The yellow circles around individual exosomes are given for visual guidance. The arrows point to the individual Au particles on the surface of a healthy exosome. C) EDX spectrum of an Au-stained P-exosome showing the Au signal. The Cu signal originates from the TEM grid.	235
Figure 6-40 Analytical performance assessment of the GFET biosensor for GPC-1+ PDAC exosome detection in blood plasma samples: A) GFET biosensor device response against the GPC-1+ exosome concentration in blood plasma (Pearson correlation 0.585; Sig. 0.002). .	237

Figure 6-41 A) Atomic force microscopy images of exosome expression on surface of 3 different GFET biosensors with different device shift after clinical testing with 3 different PDAC patient plasma samples. AFM scale bars = 1 μm . B) Exosome counts from AFM on the GFET device after clinical testing, taken from an average of four spots on each graphene surface. The GFET device shift is labelled on the chart. 237

Figure 6-42 Correlation of detection signal from the GFET biosensor with the number of exosomes binding on the GFET sensing channel. The number of exosomes is counted based on average of four different spots of AFM scans of the sensing channel on the GFET biosensor. Error bars are determined by the standard deviation of exosome counts on graphene surface ($n = 4$). 240

Figure 6-43 Detection of all PDAC cancer stages with the GFET biosensor. Measurement results of PDAC cancer stages, including stage 1, 2, 3, and 4, were classified using: A) Detection signals from the GFET biosensor; and B) GPC-1+ particle concentrations from Nano Flow cytometry measurements. 241

Figure 8-1 Schematic illustrations of the formation of electric double layer (EDL) on graphene with conventional Si/SiO₂ substrate and graphene with the hydrophobic substrate. 247

Figure 8-2 Schematics of the GFET chip for multiple biomarker detection. The chip comprises several functional areas of GFET sensor arrays. Each functional area contains the detection and negative controls for each biomarker..... 249

List of Tables

Table 2-1 Comparison of the functionalization techniques.....	55
Table 4-2 Summary of water contact angle of SiO ₂ /Si substrates with and without graphene and after surface functionalization with linker molecules. Data are mean ± standard deviation (s.d.). n = 5 for each measurements.....	144
Table 4-3 Characteristic positive ion amino acid fragments and their molar masses used for the TOF-SIMS analysis.	150
Table 4-4 Amino acid composition of F(ab') ₂ and Fc fragments from IgG and their corresponding F(ab') ₂ /Fc ratios.....	156
Table 4-5 Comparison of the prevalence of amino acids in the TOF-SIMS analysis with their composition ratios Fc/F(ab') ₂ extracted from the protein structure.....	157
Table 4-6 Adsorption energy of each PBASE configuration adsorbed on the graphene surface.	163
Table 6-1 Summary of the patient cohort.	204

1 Aims and objective

Considering the effects of the global pandemic by coronavirus disease SARS-COV-2 (COVID-19) and the high mortality rate of cancer worldwide, a simple, fast and reliable point-of-care diagnosis platform for the detection of biomarkers in physiological solutions associated with these diseases is the key to control the spread of the disease and potentially improve the treatment outcome.

Graphene field-effect transistors (GFET) have emerged as a promising platform for early diagnosis of disease due to the unique electronic properties of graphene. A graphene monolayer consists of one atom thick layer of carbon that exhibits a strong response to charged molecules present on the surface. Its extremely high surface-to-volume ratio makes graphene an ultrasensitive material to any biomolecule's interference at its surface. The low density of states (DoS) in graphene can make it easy to modify its electronic properties by coupling with charged molecules at the surface. The sensing principle relies on electrical charges expressed at the surface of molecular, cellular, or cell-vesicle based biomarkers. Once these biomarkers are immobilised at the surface of graphene, it alters the current-voltage characteristic of a graphene field effect transistor via the so-called ambipolar field effect, which results from its unique electronic band structure. In comparison to conventional field effect transistors based on silicon, the fact that graphene is just one 0.3 nm thick single layer of carbon, reduces the amount of surface charges needed to produce a detectable signal, typically corresponding to achievable limits of detection in the femtomolar of even atto-molar range in some cases. Graphene is biologically compatible and can be functionalized by non-covalent binding without the need for a reaction step or damaging its sp^2 network. The chemical compatibility of graphene with biological liquids such as blood, and the relatively simple chemistry required to functionalize graphene with antibodies or aptamers, which facilitates the capturing of specific biomarkers, makes graphene the ultimate choice among the popular "2D materials". Moreover, high quality single atomic layers of graphene with low defect density can be prepared through chemical vapour deposition (CVD) and subsequent transfer on target substrates, and industry-based foundries offer graphene transistor arrays prepared via wafer scalable processes. It also allows large scale production of GFETs sensors using standard CMOS-compatible wafer processes which can enable dramatic miniaturization

of chip footprints with microwatts of power consumption without sacrificing performance. This makes the GFET sensor platform promising for implantation at points-of-care and checkpoints or for home use.

A plethora of research results have been published about GFET biosensors demonstrating high sensitivity and high dynamic range on laboratory samples. Along with excellent device performance and ease of integration, various challenges delay the implementation of such devices in clinical practice. These include their capability to work effectively and reliably with clinical samples such as blood and urine with high ionic strength, and the possibility that non-specific binding events may influence the accuracy of the detection, the efficacy of capturing target molecules to the graphene surface. In addition, the need for bulky reference electrodes, the issues of lack of reproducibility of graphene quality, and the complex nature of surface charge-graphene interaction also hindered the practical implementation of graphene technology as a diagnostic tool.

The general aim of my PhD study is to develop a chip-based biosensor based on graphene field effect transistor biosensor (GFET) for the detection of disease biomarkers in physiological solutions. This developed GFET biosensor aimed to overcome the fundamental limitations in electrical biosensors including the Debye screening effect, non-specific binding events, and the need of bulky reference electrodes and demonstrate the capability of the platform in the detection of disease biomarkers in physiological solutions. Furthermore, the thesis aims to demonstrate the use of the platform in the detection of cancerous biomarkers in clinical samples. Exosomes, which are small extracellular vesicles (EVs) with a size range between 30–200 nm, were used for testing in clinical samples due to increasing evidence that exosomes released from cancer cells contain disease-specific information that can be used as biomarkers for diagnosis of disease at an early stage or even the prediction of disease occurrence years before its outbreak.

Thesis Outline

This thesis aims to address the significant challenges associated with the detection of disease biomarkers, particularly in the context of pancreatic cancer. Through the fabrication,

functionalization, a serial of performance optimisation, and testing of GFET biosensors, this research aims to push the boundaries of current biosensing technologies and provide novel solutions for accurate detection of specific biomarkers. The specific research objectives will be introduced and presented.

The primary objective of this study is to fabricate the on-chip integrated graphene field effect transistor biosensor (GFET). One of the key aspect in this research is to develop a new functionalisation process of graphene that can enable high capturing efficiency of target biomarkers on graphene. Through characterisation the fabricated and functionalized graphene biosensors with various characterisation techniques, a deeper understanding of the mechanism, advantages, and disadvantages of selected functionalization techniques can be obtained.

Besides the fabrication and characterization of GFET biosensors, this research also focuses on the practical application of GFET biosensors in real clinical diagnostic applications. A critical part of the study is to develop and design a testing protocol for the application of GFET biosensors for the detection of disease biomarkers in physiological solutions. This involves the investigation of the performance of functionalized graphene biosensors, particularly on its ability for the detection of exosomes as a biomarker for cancer. In this study, the performance of developed GFET biosensors are validated for the detection of cancerous exosomes in patients' plasma samples. To further study the sensing mechanism and obtain a more valuable insight on the performance of developed GFET biosensor, an analytical model is established between the GFET device response and the density of biomarkers on the graphene surface using Poission-Boltzman equations.

In this presented work, the fabrication and development of the GFET biosensors, and the characterisation of GFET biosensors using individual techniques will be performed step-by-step. A brief overview of the thesis outline is given below:

- Chapter 2 presents an overview of the basic properties of graphene. It also addresses the key challenges related to the performance limitations of GFETs in clinical settings, including sensitivity and selectivity. The strategies for enhancing the sensitivity and

selectivity of the detection of biomarkers in complex physiological conditions, including strategies to overcome Debye screening effects and non-specific binding effects, and improve signal amplification are discussed. This chapter also summarized recent studies that have successfully employed GFET biosensors in the detection of different disease biomarkers in complex mediums. Finally, the future development of GFET-based sensing systems required to enable the transition of this technology from the laboratory to clinical applications is outlined.

- Chapter 3 summarizes all characterization and analytical techniques used in this thesis. The experimental methods and materials used for this thesis are also presented. For the GFET biosensors, the steps for the fabrication and functionalisation of the GFET sensor will be introduced. For the characterisation of fabricated and functionalized GFET sensors, the basic principles of the analytical characterisation techniques selected are described.
- Chapter 4 is the first chapter of the results. It presents the study of the orientation of antibodies on graphene surface functionalised using two different linker molecules, conventional 1-pyrenebutanoic acid succinimidyl ester (PBASE) and the new biofunctionalization linker tetrakis (4-carboxyphenyl) porphyrin (TCPP). Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) and Density-functional theory (DFT) simulations were employed with rigorous analyses. It is found that the newly developed biofunctionalization protocol with TCPP showed a predominant end-on vertical orientation of the Immunoglobulin G (IgG) antibody on functionalized graphene, as opposed to the varied orientations on PBASE-functionalized graphene.
- Chapter 5 is the second chapter of the results. The performance of the developed GFET biosensors functionalized with the TCPP method and the PBASE method is further evaluated in this chapter. Electrical measurements are performed in this chapter. Several unique designs, including the use of on-chip integrated electrode, differential measurement with separated control and sensing channel, and the use of a house fabricated microfluidic channel are implemented and tested. This study demonstrated a two orders of magnitude improvement in the limit of detection for the TCPP-functionalized GFETs, with a threefold surge in exosome density on the graphene surface.
- Chapter 6 is the final chapter of the results. The performance of the developed TCPP-functionalized GFET biosensor is further validated with clinical blood plasma samples from

26 PDAC patients and healthy controls. In this chapter, the uniformity test, sensitivity test and selectivity test were performed to characterize the GFET biosensors. The developed GFET biosensor can clearly discriminate the PDAC patient and the healthy controls. Further characterizations on the exosomes derived from the blood plasma samples are also performed. The unique characteristics of cancerous exosomes with the higher density of GPC-1 proteins expressed on cancerous exosomes compared to healthy ones are demonstrated. This further proves the capability of developed GFETs as an ideal and promising platform for the detection of pancreatic cancer exosomes. An analytical model is also established using Poission-Boltzman equations to fit the GFETs response with the density of exosomal biomarkers captured.

- Chapter 7 is the final chapter of this thesis. It summarizes all the work carried out and presented. A future perspective outlook is also presented in this chapter.

The key findings/novelty in this thesis:

The thesis attempts to address the key challenges in detecting disease biomarkers in clinical setup and improve the accuracy of the detection of disease biomarkers in clinical samples. Therefore, the key novelty is the demonstration of the detection of pancreatic cancer biomarkers in clinical setup using GFETs biosensors. The thesis advances our understanding of the biosensor field through several different aspects. Firstly, the research advances the field by enhancing the capturing efficiency and performance of GFETs by optimisation of the functionalization process. Moreover, the investigation of the orientation of antibody on graphene using TOF-SIMS and DFT simulation provide a more comprehensive understanding of antibody orientations on graphene. A specific achievement of this research is the improvement of the sensitivity and limit of detection (LOD) of GFETs, an improvement of more than two orders of magnitude in the detection limit is realized.

Additionally, the development of testing measurements and protocols enables accurate measurements in physiological samples and reduces device drift and non-specific bindings. Ultimately, this thesis demonstrates the detection of pancreatic cancer biomarker in patients' plasma samples using GFETs and validation using a large number of on-chip GFETs sensors.

2 Biosensing using graphene opportunities and challenges

Rapid, simple and low-cost detection of disease biomarkers in easily accessible biofluids such as saliva, nasopharyngeal swabs, blood, and urine currently represents the most promising approach for early detection of common diseases like cancer, neurodegenerative and cardiovascular, as well as virus infections. Regular screening of large numbers of individuals in point-of-care settings could enable more targeted early interventions and therapies.[1], [2], [3] At the same time, the global threat of infectious diseases requires a flexible, rapid and low-cost sensor platform for the detection of viral infections. [3], [4], [5]

Current methods for the detection of biomarkers in liquid biopsy samples include, polymerase chain reaction (PCR) for the analysis of genetic contents, enzyme-linked immunosorbent assay (ELISA) for protein detection, ctDNA analysis, and so on. However, these techniques usually involve complex and expensive laboratory procedures, with specialized equipment and trained personnel required, and may be lack of adequate LoD for the detection of early-stage disease. More recently, many sensor technologies, including electrochemical sensors, lateral flow assays, and surface-enhanced Raman scattering (SERS) based sensors, have been proposed and developed for the detection of these disease biomarkers in the liquid biopsy samples [6], [7], [8], [9].

Biosensors based on nanomaterials have shown excellent sensing performance to meet medical demands for better healthcare. [10], [11], [12] Among them, biosensors based on graphene field-effect-transistor (GFETs) are especially promising and could play a key role in future diagnostic applications. GFET biosensor has distinct advantages over traditional liquid biopsy methods and biosensing technologies. Graphene is a single layer of carbon atoms arranged in a 2-dimensional hexagonal lattice structure. Graphene exhibits exceptional properties, including low density of states, high electrical conductivity, large surface area, high biocompatibility, and low intrinsic noise. These properties make graphene extremely sensitive to all surrounding electrostatic charges and ideal for biosensor applications. A detailed introduction to graphene and graphene field effect transistor (GFET) will be presented in section 2.1.

The ease of functionalization gives GFET biosensors many possibilities for the detection of various biomarkers. The surface of graphene can be easily functionalized covalently or non-covalently for the immobilization of bioreceptor molecules. Through the functionalization of graphene surfaces with different bioreceptor molecules, such as antibodies, aptamers, and other probes, GFET biosensors can be tailored and easily adapted for the detection of a wide range of biomarkers. [13], [14] The versatility of GFET biosensors in the choice of bioreceptor molecules empowers researchers to design GFET biosensors for various applications, enabling multiple detection with a single device. Details about the functionalization of the GFET biosensors will be discussed in section 2.2.2 and section 2.2.3.

Besides, another key advantage of GFET biosensors lies in their scalability for production and fabrication. Graphene can be synthesized, and mass-produced at a large scale, enabling the fabrication of GFETs using an 8-inch wafer technology.[15], [16] This provides the route for the cost-effective mass production of GFET biosensors. [17]

Moreover, the GFET devices can be multiplexed. [18] [19] This feature allows the simultaneous detection of multiple biomarkers in the same sample from liquid biopsy. This could not only significantly improve the accuracy and efficiency of diagnostics, but also reduce the number of samples required and turn-around time, making GFET biosensors particularly useful for the POC applications. The multiplexed GFET biosensors holds great promise for the detection and monitoring of complex diseases that relies on the change from multiple biomarkers.

The technology of on-chip integrated GFET biosensors further enhances the convenience and ability of GFET biosensors for POC applications. GFET biosensors can be integrated with portable electronics, enabling real-time data acquisition and analysis [17], [20].

Along with excellent device performance and integration, various challenges could delay the implementation of such devices in clinical practice. These include their capability to work effectively and reliably with clinical samples such as blood and urine with high ionic strength, and the possibility that non-specific binding events may influence the accuracy of the detection. In this chapter, the methods of functionalisation of graphene to transform into a biosensor were first highlighted. Then, the key challenges in incorporating GFET biosensors in clinical settings and many innovative methods and techniques used to enhance device

performance in physiological solutions were discussed. In addition, recent studies that have successfully employed GFET biosensors in the detection of various disease biomarkers in complex mediums were discussed.

This chapter is part of the review paper publication: Tianyi et al. "Progress in graphene field-effect biosensors for the detection of disease biomarkers in physiological solutions" in progress.

2.1 Introduction to graphene and graphene field-effect transistors

2.1.1 Electronic properties of graphene

Monolayer graphene was first isolated in 2004 by Geim and Novosolov. [21] It is a single layer of carbon atoms arranged in a two-dimensional hexagonal lattice structure with an interatomic distance of $1.42 \text{ \AA}$ between its nearest neighbours. The thickness of a single layer graphene is one atomic layer thick, which is 0.335 nm . Graphene is a zero-band gap material, with its conduction and valence band meeting at the Dirac Point, within the Brillion zone. [21] Graphene has unique properties, including extremely high mobility ($10^5 \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$), low density of states, extremely high surface to volume ratio, and low intrinsic noise. Graphene has been studied and investigated for application in various fields, including electronics, biosensors, energy storage, photonics, and flexible electronics. [22], [23], [24], [25], [26]

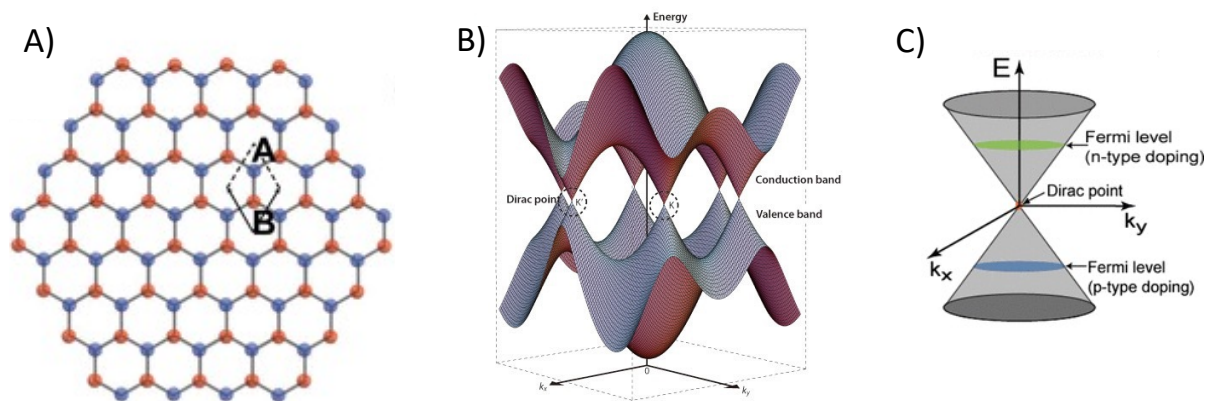


Figure 2-1 A) Hexagonal lattice of graphene, two atoms (A and B) per unit cell. [27] B) Electronic structure of graphene: Energy bands near the Fermi level in graphene. The conduction and valence bands cross at the points K and K'. [28] C) Energy band of graphene, the conduction and valence bands touching at the Dirac point. The position of the Fermi level determines the nature of the doping and the transport carrier. [27]

2.1.2 Graphene growth and fabrication

To fabricate a graphene-based biosensor, three major steps are required: 1) graphene synthesis, 2) graphene transfer, and 3) metallic contact deposition and encapsulation.

Graphene can be synthesised via many methods, including exfoliation, chemical vapour deposition (CVD), and epitaxial growth, etc.. [29] The exfoliation of graphene is the process that separates the graphite into layers of graphene. There are various methods developed for the exfoliation of graphene, such as liquid-phase exfoliation, mechanical exfoliation, electrochemical exfoliation, and micromechanical exfoliation. [30], [31] The liquid phase exfoliation is a promising approach for the scalable production of graphene with high efficiency and low cost. The micromechanical exfoliation of graphene involves the separation of graphene from the graphite using an adhesive tape or scotch tape. Typically, the graphene obtained from micromechanical exfoliation has the best electrical properties. However, the exfoliation of graphene is not able to provide large area graphene, which is less suitable for the large scale mass production of defect free graphene devices. [30], [31]

Chemical vapour deposition (CVD) is one of the most widely used method for the production and synthesis of large scale and large area graphene devices. CVD is a process where a solid material is deposited from a gaseous phase using a chemical reaction between the volatile precursors and the surface of the heated substrate surface. The CVD graphene is most commonly grown on metal substrates, various metals have been studied, such as Ni, Cu, Co, Au, and Ru. [29], [32], [33] Nickel was the first attempted substrate for the CVD growth of graphene. Copper (Cu) is the most popular substrate for CVD graphene, it allows the continuous growth of monolayer graphene without introducing significant defects. This is because the solubility of C in Cu is low, therefore the carbon growth on the copper substrate is a self-limiting process. [33] During the CVD process, methane is the most common hydrogen gas source, acetylene and ethylene can also be used. Hydrogen gas is also broadly used in addition to promoting the reaction, however, the use of hydrogen may alter the structure of graphene and introduce additional defects. [32], [34], [35] The growth temperature plays an important role in the quality and orientation of CVD graphene. It is found that a high growth temperature, close to the Cu melting temperature (1083 °C), is preferable for the production of high-quality graphene. Through careful control of the reaction parameters, including the gas source, the ratio of H₂:CH₄ gases, the reaction pressure, and the growth temperature, the quality of graphene growth can be adjusted. [34], [35], [36] The main steps involved in the CVD process are 1) the dissociative adsorption of CH₄ gas once it reaches the proximity of the

Cu surface and bind to Cu surface sites, 2) the desorption of adsorbed hydrogen, 3) graphene formation from carbon adsorbed onto the Cu surface. (Figure 2-2 B))

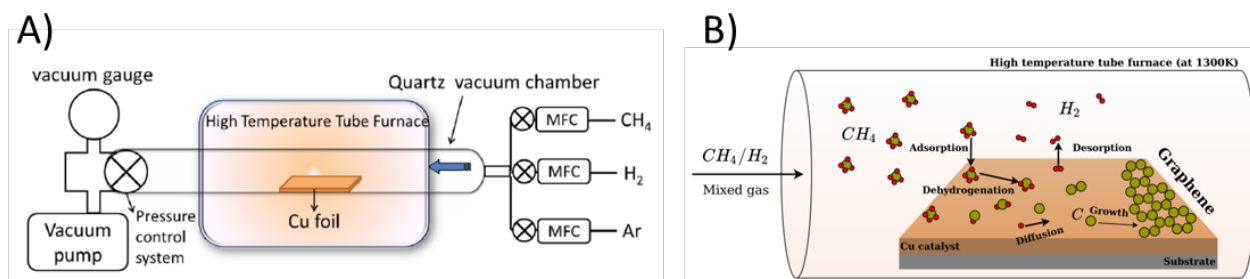


Figure 2-2 A) Schematic of the CVD system.[16] B) Schematic diagram of the process of chemical vapor deposition procedure for graphene synthesis with Cu.[37]

Following the growth of graphene, the layer of graphene is required to be transferred onto the desired substrate. The selected substrate needs to provide physical support to the thin layer of graphene while avoiding unwanted electrical connections between the electrodes deposited on the graphene. The selected substrate should be a dielectric material or other insulating material. It is challenging to transfer the thin 2D graphene film onto the substrate without damaging and introducing defects to the graphene film. This is because the properties of graphene are highly affected by the defects, impurities and wrinkles within the graphene. Multiple techniques have been developed for graphene transfer, including wet transfer methods and dry transfer methods. [38], [39]

The wet transfer method is performed with a protective layer of poly(methyl methacrylate) (PMMA) on top of the graphene. Then the growth substrate, typically Cu, will be etched in an aqueous Cu etchant. The PMMA protected graphene will then be transferred onto the desired substrate and dried. Finally, the PMMA layer will be removed. Although this method is the most widely used method for graphene transfer, the PMMA residue will alter the electrical properties of graphene and change the doping of graphene, shifting its Dirac point to the left. [40], [41].

The dry transfer method is performed with hot pressing [42], roll-to-roll method [43] or pick-up and stamping with PDMS [44]. The dry transfer is based on the van der Waals interactions.

The delamination of graphene is achieved by introducing intermediate layers on the surface of graphene. This intermediate layer has higher binding energy than the metal substrate. The intermediate layers used can be easily removed by water. It is believed that the dry transfer method allows the transfer of larger area and high-quality graphene. However, cracks are easily formed during the delamination of graphene. The dry transfer method still needs to be carefully optimized for the larger scale production and commercialization. [45], [46]

2.1.3 Graphene field effect transistor biosensor basic principle

A field effect transistor is a transistor that uses the applied electric field and its associated voltage to control the current passing through the semiconductor. It is a three-terminal device, which is consisted of source, drain and gate electrodes. A semiconductor channel is placed between the source and the drain, the channel is either a p-type or an n-type semiconductor. Through an applied voltage to the gate, the gate voltage can control the flow of current and in turn the conductivity in the semiconductor channel.

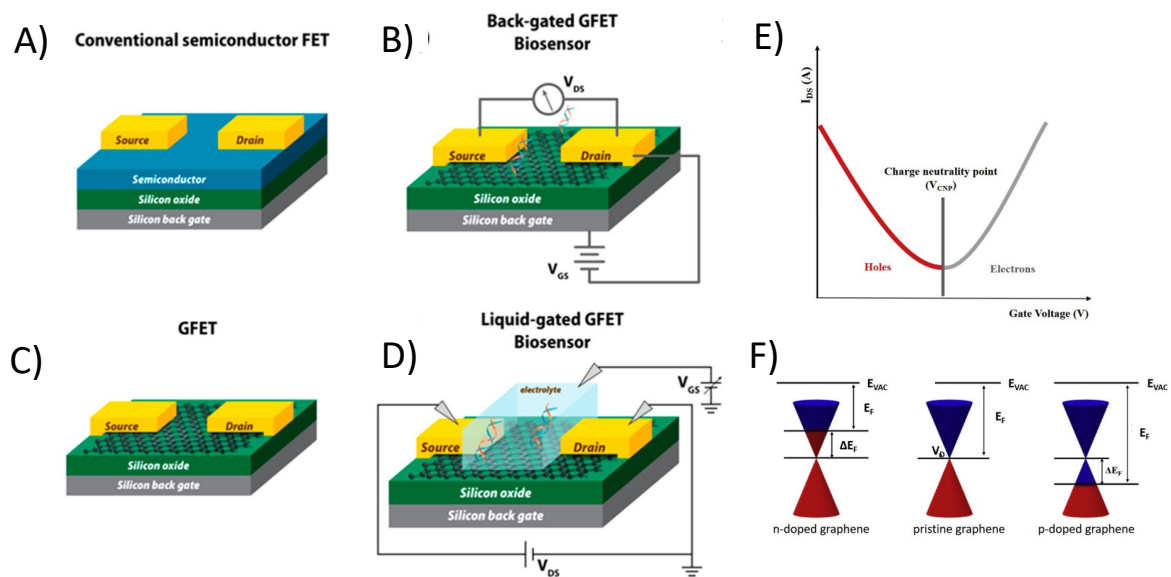


Figure 2-3 Schematic diagram of A) a conventional FET device, B) a back gated GFET device, C) a GFET device, D) a liquid gated GFET device. [47] E) IV characteristic diagram of graphene's unique ambipolar effect with two types of charge carriers in graphene. The field effect can modulate the holes (red colour, in left) to electrons (grey colour, in right). The charge-neutrality point V_{CNP} is located at the transition between the hole and electron regime, where the current is at its minimum. F) Schematic diagram of the modulation of the Fermi level position as the function of doping, with n-type doping, pristine and p-type doping graphene.[48]

Graphene field effect transistors (GFET) work similar to other types of field-effect transistors with one layer of graphene channel between the source and drain electrodes. The potential difference applied between the gate and drain can modulate the doping of graphene. When the gate voltage in a GFET device is changed from positive to negative, conduction switches from electrons to holes. This is known as graphene's ambipolar field effect. (Figure 2 E and F) The device can be used to sense chemical doping of the surface, which results in a change of the Dirac Point voltage. Comparing to conventional FET biosensors with bulk materials, such as silicon, GFET biosensors offer significant advantages. These advantages of GFET biosensors include high sensitivity, faster response time, and lower detection limits.

2.2 GFET biosensors

2.2.1 Sensing mechanisms

GFET biosensors are three-terminal devices that consist of source (S), drain (D), and gate (G) electrodes. The gate electrode is important as a potential regulator setting the operating regime of the sensor. The GFETs with the liquid-gate is the most suitable gate configuration for biosensing. The use of liquid gate allows the replication of the natural environment for biological species. Also, it provides a stable potential and enables the detection at low operating voltage. The source/drain electrodes are isolated from the solution using a passivation material such as aluminium oxide (Al_2O_3), silicon nitride (Si_3N_4), silicon dioxide (SiO_2), and SU8. GFET biosensors use the charge as a primary physical quantity of any target. The change in surface potential across the electrolyte-graphene surface due to molecule binding results in a change in the conductance between the source and drain. This can be

reflected by the change in the direction and amplitude of the transfer curve of the GFETs (Figure 2-4).[49], [50] The curve is typically a V-shaped curve due to the ambipolar characteristics of graphene transistors resulting from the band structure of graphene. This allows graphene to conduct both electrons and hole current depending on the position of the Fermi level.[49], [50], [51] Therefore, the binding of biomolecules in the graphene surface can cause the transfer curve to shift to the left or right, depending on the charge of the biomolecule. GFET biosensors benefit from high sensitivity, selectivity, biocompatibility, ease to functionalize, manufacture in mass quantity, and low operating voltage.

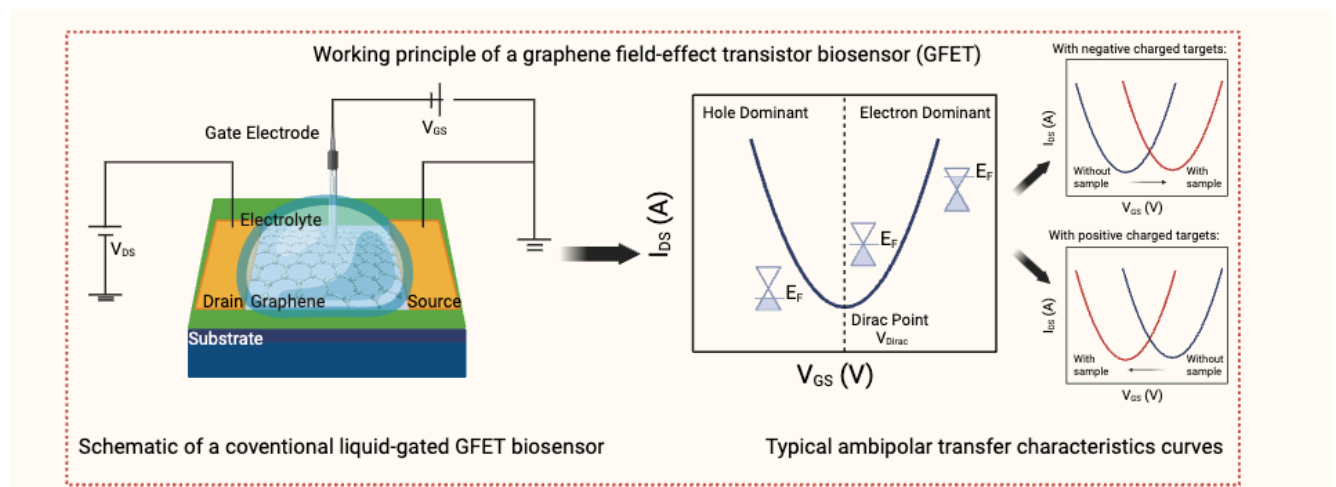


Figure 2-4 Graphene field-effect-transistor as a versatile platform for biomarkers detection. GFETs biosensor structure and its typical transfer characteristics. The binding of charged molecules on the graphene surface leads to a change in the Fermi level with the respect of Dirac point. Negative charges p-dope graphene lead to a right shift in the transfer curve, while positive charges n-dope graphene leads to a left shift.

GFET biosensor consists of three main layers, as illustrated in Figure 2-5. The first layer is a single layer of graphene, and then a functionalization layer of linker molecules is placed on top of the graphene via covalent or non-covalent bonding. These linker molecules allow the graphene to immobilize a bioreceptor layer to bind with the target biomolecules in a sample or specimen. Depending on the target biomolecules, the bioreceptors can be antibodies, aptamers, ssDNA and enzymes. When the target molecule binds with the immobilized bioreceptors on graphene, the current-voltage characteristic, also known as the transfer characteristic of GFET will be altered via graphene's unique ambipolar field effect. [7][8] The

biofunctionalization of the GFET biosensors will be introduced in detail in the following section.

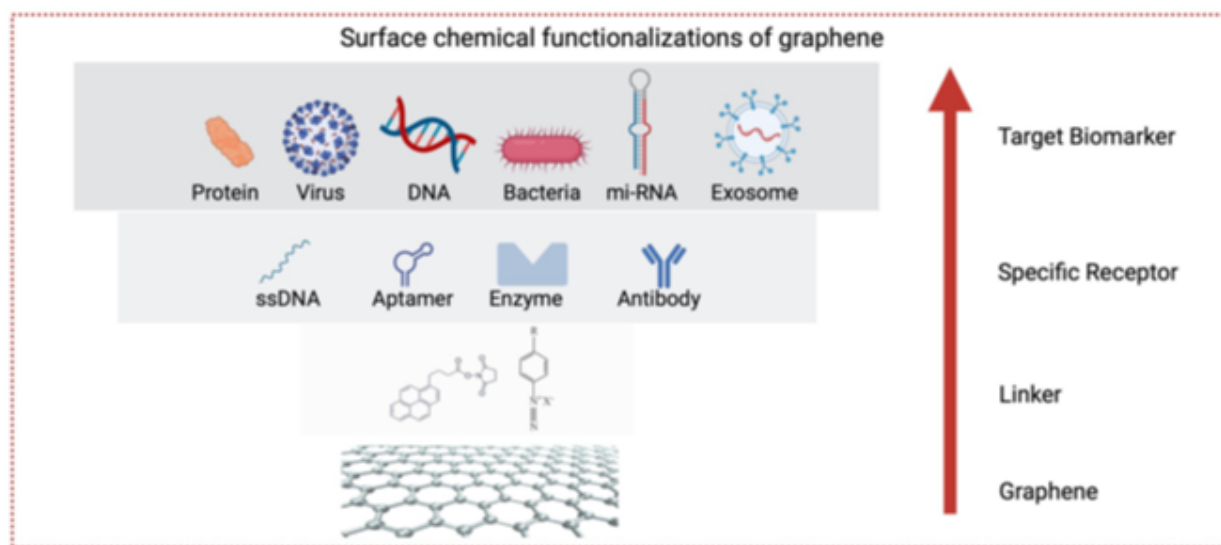


Figure 2-5 Schematic representation of chemical functionalization of graphene surface for various range of biomarkers. The universal surface functionalization technique allows graphene based biosensors for the detection of a wide range of target biomarkers using chosen specific bioreceptors. All biomarkers are not present in scale.

2.2.2 Biofunctionalisation of graphene field effect transistors

Biofunctionalisation of graphene surface is the key step to transform GFETs into biosensors and enable its use in a wide range of biosensing applications. It allows the selective and specific recognition of target molecules on the graphene surface. Covalent and non-covalent functionalisations are the two main strategies for GFETs biosensors. The covalent functionalisations are generally achieved via the attachment of specific functional groups onto the surface of graphene through covalent bonding, while the non-covalent methods are based on π - π interactions between graphene and aromatic groups in the crosslinker. In order to achieve optimum biosensor performance, the surface functionalisation approach needs to meet certain standards. First, the biorecognition molecules should be attached uniformly and evenly to the sensor surface while preserving their biological properties. Second, the functionalisation process should enable high coverage and proper conformation of receptors at the graphene surface. Third, the functionalization process needs to be highly reproducible.

Fourth, the surface or grafting process should be tunable to achieve a higher degree of functionalisation. Fifth, the biofunctionalisation process should preserve the quality of graphene.

2.2.2.1 Covalent functionalisation

Graphene is a stable planar sheet of sp² carbon atoms in a tightly bound hexagonal lattices.[54] Therefore, it requires high energy to introduce the desired functional groups onto graphene. The formation of covalent bonds between the functional groups and graphene provides a strong attachment, minimizing the risk of detachment.

The main reactions for the covalent functionalization of graphene are free radical reactions, cycloaddition reactions, and electrophilic substitution reactions. Due to the low reactivity of tightly bonded graphene basal plane, highly active free intermediates, such as strong acids or free radicals, are required to form covalent bonds with the carbon atoms in the graphene lattice. [55], [56], [57]

The use of diazonium salts is one of the most extensively studied and widely employed chemistry for covalent functionalization of graphene. A diazonium salt, as illustrated in Figure 2-, is consist of a para-substituted functional group R. Through coupling the R functional group with biorecognition molecules, graphene can be used as a sensor for various biological analytes. [55], [58], [59] Various diazonium salts with different functional groups are available for grafting the organic molecules onto the graphene sheet. -NH₂ and -COOH are the most frequently used R groups. These functional groups are compatible with EDC/NHS chemistry for the formation of -CO-NH- bonds with the biorecognition molecules, including antibodies and aptamers. [50], [59] While EDC/NHS chemistry is the most popular approach for crosslinking of linkers with biorecognition molecules, there are still some drawbacks, including random coupling sites and crosslinking during immobilization. Click chemistry is considered to be an alternative and more advanced immobilization technology. Mishyn et al. [60] reported the option of 4 [(triisopropylsilyl) ethynyl]-benzene diazonium tetrafluoroborate (TIPS-Eth-ArN₂⁺) modified GFET biosensor, with monolayer coverage and maintaining good electron and hole mobility. The formation of ethynyl phenyl monolayer on

graphene allows the immobilization of biorecognition molecules using click chemistry. [60] T. Rodrigues et al. [61] further demonstrated the ability of TIPS-Eth-ArN²⁺ modified GFET biosensor with aptamers for specific sensing of cardiac troponin I (cTnI) in different bodily fluids.

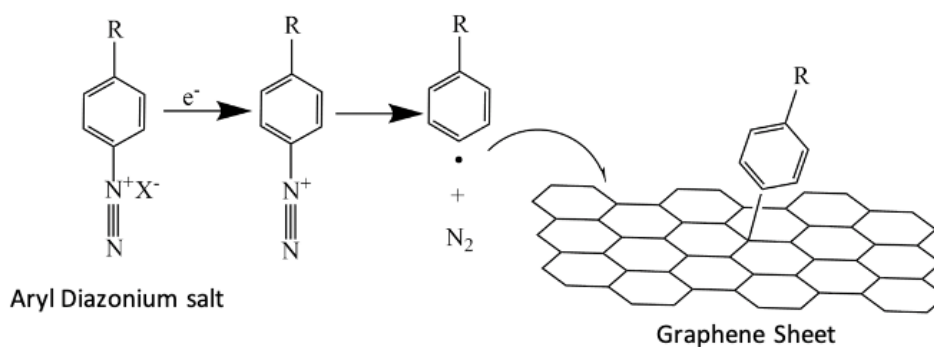


Figure 2-6 Schematic of the structure of a diazonium salt and process of covalent attachment of a diazonium salt to the graphene sheet surface.

There are some challenges associated with the covalent functionalization of graphene. During covalent modifications, the free radicals will activate the point defects in graphene [62], and transfer electrons between the free radical and carbon atom in graphene. [55], [56] Meanwhile, it transits the hybridization state of carbon atoms in graphene sheet from sp^2 to sp^3 , and displaces the carbon atoms out of the graphene plane by around $0.7 \text{ \AA}$. [58], [60] This disruption alters the electronic properties of graphene, including its conductivity, charge mobility, and even band gap. [50], [63] Most studies reported that the covalent grafting of diazonium salts decreases the conductivity and charge mobility of the graphene sheet. [57], [60], [64], [65], [66], [67] This is because during covalent functionalization, the change of hybridization state of carbon atoms and the introduction of defects in the graphene sheet result in a reduction in the number of conductivity electrons and the generation of charge scattering sites. [68], [69] Consequently, lower the electrical conductivity of covalently functionalized graphene.

It is crucial to achieve a monolayer of grafting species for the optimum degree of functionalization. [72], [73], [74] Low degree of functionalization [58], [72], [75], [76], [77], [78] and formation of oligomers or multi-grafted layers [56], [58], [66], [79] is generally

reported for covalent functionalized graphene surface. Since the aryl radicals are highly reactive, side reactions of radical attachment on the existing chemical groups on graphene rather than the graphene basal plane are likely to happen. [79] As a result, oligomers or multi-grafted layers are formed, as illustrated in Figure 2-7. [11][12][18] Because of the steric repulsion between two adjacent bulky phenyl groups on the same graphene sheet, these oligomers strongly hinder the packing uniformity and efficiency of bonded groups on graphene.

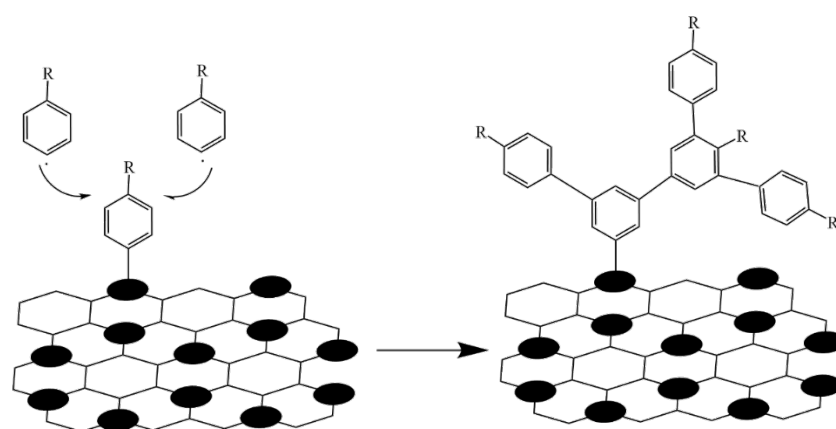


Figure 2-7 Schematic illustration for the process of covalent grafting of aryl diazonium radicals on graphene leading to oligomer formation. Side reactions of radical attachment on the existing chemical groups on graphene rather than the graphene basal plane are likely to happen during covalent functionalization process with aryl radicals.

Research has been conducted to investigate the surface coverage of the covalently attached groups. 25% is by far the highest simulated [12][26] as well as experimental [27-29] maximum coverage of the attached groups, as shown in Figure 2-8, while most studies reported a maximum coverage of around 10-20%. [27][30] These will reduce the binding efficiency of target biomolecules in solutions. In turn, it affects the overall performance of the fabricated graphene biosensors, particularly their sensitivity. Therefore, it is important to form a monolayer of grafting species for the optimum degree of functionalization. Extensive studies have been conducted to find the approach for monolayer grafting on the graphene surface. [72] Greenwood et al. reported that through the application of bulky 3,5-bis-tert-butylbenzenediazonium (3,5-TBD), a relatively higher grafting density can be achieved with monolayer coverage. [73] Nguyen et al. achieved the growth of a self-organized monolayer

of aryl diazonium salts on graphene via electroreduction. During their grafting process, a small electrochemical bias potential is applied. [74] These studies indicate the selection of novel methods and techniques is essential to produce monolayer grafting for a maximum degree of graphene functionalization, leading to improved sensitivity and selectivity for the detection of target biomarkers.

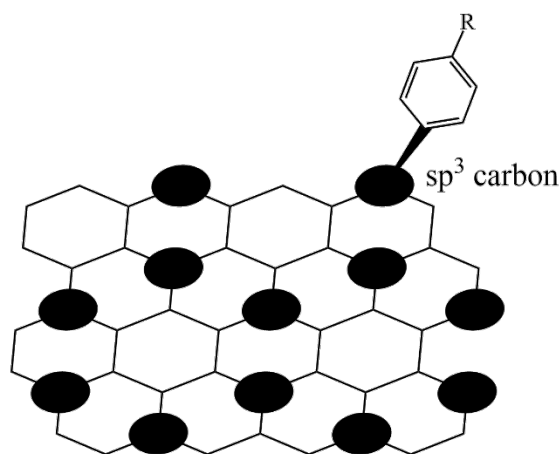


Figure 2-8 Schematic demonstration of maximum theoretical coverage of covalently attached diazonium salt group on graphene.

The contribution from covalent modification to the electronic properties of graphene and its degree of functionalization strongly depends on the reaction conditions, such as the concentration of reagents and reaction time. [60], [67], [71], [80], [81], [82] The reaction needs to be carefully optimized to preserve the conductivity and charge mobility of the graphene sheet and to achieve the optimum degree of functionalization, thereby improving graphene sensing sensitivity and selectivity for target biomarkers.

2.2.2.2 Non-covalent functionalization

Non-covalent functionalization of graphene biosensors is based on π - π interactions between graphene and aromatic groups in the crosslinker. This functionalization method allows for the generation of a self-assembled, homogeneous functionalized graphene surface. This strategy can preserve both the aromaticity and the electronic property of graphene,[63], [68] making

it particularly attractive to biosensor applications. Retaining the high mobility of graphene is particularly advantageous for developing fast and sensitive GFET biosensors. Various crosslinkers, including pyrene-based molecules [50], [63], [68], polymers [83], and peptides [84], are available for non-covalent immobilization on graphene. 1-pyrenebutanoic acid succinimidyl ester, known as PBASE, is the most widely used pyrene-based crosslinker for immobilization of biorecognition molecules on graphene. [17], [18], [50], [63], [68], [85], [86], [87], [88], [89], [90], [91], [92], [93], [94], [95] The structure of PBASE is illustrated in Figure 2-. Its pyrene group interact with the graphene surface via Van der Waals force, forming a π - π bond. Then, the N-hydroxysuccinimide (NHS) ester group at the other end of PBASE can react with primary amines or lysine in biomolecules, forming an amide bond. PBASE can be assembled on graphene surface through drop-casting or immersion methods. [85] The formation of a monolayer of PBASE on the graphene surface is a self-limiting process. [96] The large NHS ester group on the other end of PBASE can hinder the second layer of molecules from aggregating on top. [96] The formation of monolayer of PBASE on the graphene surface is important in GFET biosensing because it enables strong electrostatic coupling between biomolecules and graphene. [86], [96] As GFET detection of biomolecules in electrolyte solution is limited by the response to the charges located within Debye length, the thickness of the crosslinker can influence the sensitivity of the sensor. It has been experimentally observed weaker electrostatic coupling and reduction in GFETs response with the increase of thickness of receptors on the surface graphene. [63]

It is necessary to achieve high surface coverage without degradation of graphene properties after PBASE to obtain a large response from a GFET. Many research works use 1-2 h incubation time to grow PBASE on the graphene surface. However, Wang et al. identified the optimum incubation time for graphene with PBASE in DMF is 4 hours, but this long incubation time of PBASE on graphene leads to an increase in defect formation with a structure similar to bi-layer graphene. [97] Furthermore, the possibility of ester end hydrolysis with a long PBASE incubation time is likely to happen. This may lead to a lower density of available sites for grafting of biorecognition molecules. [63]

Although PBASE is currently the most popular crosslinker for biorecognition molecule immobilization, the optimum reaction condition for PBASE and whether it is the best option

when compared to PBASE and other monovalent pyrene derivatives. [101], [105] These findings imply the potential of constructing a robust and stable graphene-based sensing platform using tripodal functionalization. However, there is no systematic study related to the performance of this functionalization technique as a biosensor, with no further study conducted on tripodal modification of single layer graphene-based biosensors.

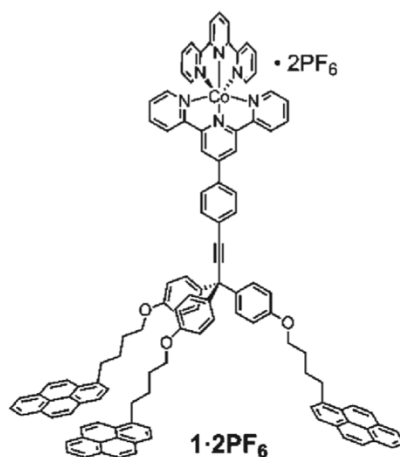


Figure 2-10 Schematic diagram of NHS ester tripod linker, with three pyrene "feet" for interaction with graphene sheet. [101]

2.2.2.3 Comparison between covalent and non-covalent functionalization technique

The performance of GFET biosensor strongly depends on the chosen functionalization technique. It is crucial to maintain the superior electronic properties of graphene while achieving the optimum degree of functionalization for higher sensitivity, selectivity and stability of the device. Covalent functionalization has the ability to construct robust and stable surfaces for biosensors. [60] However, the sp^2 to sp^3 hybridization of carbon atoms and the number of grafting layers on graphene can lead to a reduction in the electronic properties of graphene. The degree of functionalization and electronic properties of functionalized graphene sensors strongly depend on the reaction conditions and the chosen chemical reagent. To achieve high quality functionalized graphene devices, it is important to use freshly synthesized, high purity diazonium salts, because the diazonium salt will decompose over time. [70] Meanwhile, it is important to use a carefully optimized method to achieve

monolayer grafting and optimum functionalization density. Consequently, the covalent grafting process is complex and often requires several days for multiple steps and strict reaction conditions.

Non-covalent immobilization of biomolecules offers a simple and cost-effective approach that can maintain the crystal structure of graphene. However, it suffers from low stability of the immobilized surface and the process lacks robustness. It is challenging to control the number of immobilized linkers on graphene. It is widely believed that non-covalent grafting approach is by far the most promising technique for achieving high performance biosensors. In particular, there is a clear trend of using PBASE for functionalization of graphene biosensors. [17], [18], [50], [63], [68], [85], [86], [87], [88], [89], [90], [91], [92], [93], [94], [95] However, there is a lack of systematic studies comparing covalent and non-covalent modifications of graphene-based biosensors. The effects of different surface grafting techniques on the fabrication of reliable, reproducible, stable and sensitive graphene-based biosensor still needs to be thoroughly studied.

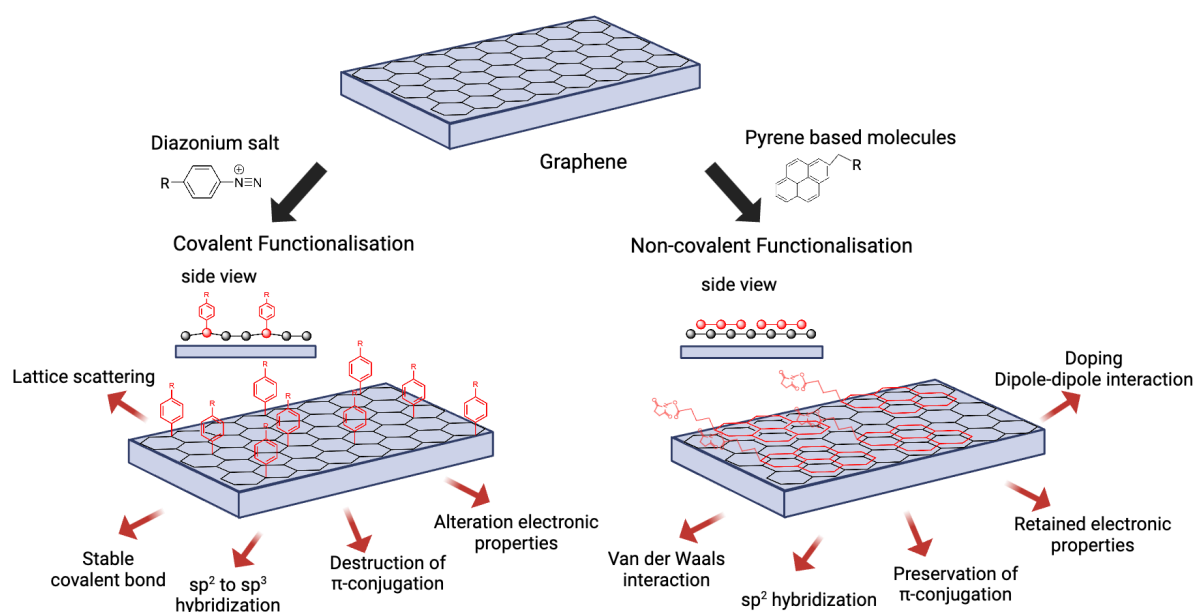


Figure 2-11 A schematic representation of different functionalization processes used in graphene biosensors and their effects on the structural and electronic properties of graphene.

Table 2-1 Comparison of the functionalization techniques

Functionalization Category	Characteristics	Advantages	Drawbacks	Interactions	Examples (Intermediates) and characteristics
Covalent	Introduction of functional groups to the C-C network; Reaction with the sp² carbon, rehybridize them to sp³ carbon; Free radicals attack the graphene basal plane; Organic functional groups react with the oxygen containing groups of GO or edges of graphene.	Strong chemical bonds; Stable and robust	Long preparation and processing time; Disruption of graphene sp² network, loss of the electronic property; Need careful optimization of reaction condition	Free radical addition reactions; Nucleophilic addition; Cycloaddition reactions; Electrophilic substitution reactions.	4-carboxybenzenediazonium tetrafluoroborate (CBDT); 4 [(triisopropylsilyl) ethynyl]-benzene diazonium tetrafluoroborate (TIPS-Eth-ArN₂⁺); 7,7'-dimethoxy-1,1'-binaphthalene; azomethine ylide; 2,3-dimethoxybutadiene (DMBD); Nitrenes; benzoyl peroxide (1,2) (3-7)
Non-covalent	Van der Waals forces; π-interactions	Do not alter the structure of graphene; Preserve the electronic property of graphene ; Simple and cost-effective	Weak interactions; Less stable, Short shelf life;	π-πstacking; Hydrophobic interactions	Pyrene-based molecules (PBASE, PBA, PMAL) Porphyrins; NHS ester tripod motif; Polymers; Peptides (8-10) (11) (12) (13) (14)

2.2.3 Selection of bioreceptor molecules

Graphene based biosensors can be applied in many areas, including the detection of pH, blood sugar level, protein, micro-organism, and nucleic acids (both DNA and RNA). High selectivity of a biosensor is achieved by binding between the specific recognition receptor molecule and the target ligand molecule. Therefore, the selection of a suitable bio-recognition molecule plays a critical role in generating sensitive and selective biosensing platforms. There are several different types of bioreceptor molecules, such as antibodies, aptamers, DNA molecules, enzymes, etc. [106]

Antibodies are the most common used biological recognition molecule for the detection of proteins, including proteins in cancer biomarkers [44][45], surface bacteria [46][47], viruses [48-50], exosomes [22][37], and other molecules [51-53]. An antibody is a roughly a 15 nm Y-shaped molecule, with four polypeptides and amino acid sequences in the Y tips. The structure of an antibody is demonstrated in Figure 2-. A biosensor platform with antibody immobilization is attractive due to the specific high affinity conjugation between antibody and antigen. [119] However, antibody-based biosensor suffers from problems including easy degradation, low stability and high costs. Meanwhile, the size of the antibody is large, which exceeds the Debye screening lengths in physiological solutions.

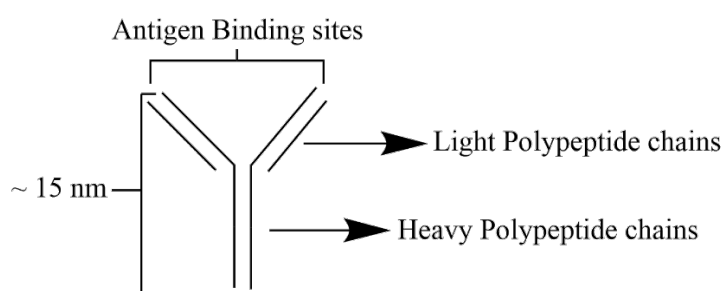


Figure 2-12 Schematic diagram for shape of antibody.

To address this limitation, researchers have explored the use of smaller bioreceptor molecules, such as antibody fragments (82,85), nanobodies (86–88), and aptamers (36,89–

92). These smaller bioreceptors allow the biorecognition reaction to occur at a closer distance to the sensor surface, enabling improved sensitivity in GFET biosensors. The binding affinity between bioreceptor molecules and target molecules is another critical factor in achieving high sensitivity in GFET biosensors. Aptamers offer an alternative advanced solution as a surface probe molecule. Aptamers are artificial single strand oligonucleotides that can bind to target molecules through shape recognition. The application of biosensors with aptamers immobilized as probes is rapidly emerging. [17], [92], [104], [120], [121], [122], [123], [124], [125] This is because the aptamers can be easily prepared and stored, offering the possibility of mass production and good reproducibility. [126] They are not affected by environmental factors, such as humidity or temperature. [54][56] Moreover, aptamers have a simpler structure with nucleic acids, thus higher stability than antibodies (with amino acids). [128] Aptamers can be denatured and refolded multiple times without losing their activity. [129] It is reported that the fabricated aptamer-based biosensor can retain its sensitivity and stability for around one week. [127] Aptamer-based biosensors can be tailored for specific detection of a range of targets, including nucleic acids [130], ions [131] and lipids [132], which are not possible to be detected by antibodies. [62][63] Aptamers are especially useful for graphene field effect transistor biosensors due to their small size (~2nm). It is believed that the small size of aptamer will help with the reduction of the Debye screening effect, thus leading to higher sensitivity of biosensors. [56][64] However, there are still some limitations in aptamer-based biosensors, such as the complex production process of aptamers and the limited availability of aptamers for targets. [135]

The effect of the selection of different biorecognition molecules is further addressed in the performance metrics section.

2.3 Performance metrics and limitations in graphene field effect transistors

2.3.1 Challenges in Real Clinical Physiological Solutions

Sensitivity and selectivity are the major challenges that need to be addressed when testing biomarkers in physiological solutions in clinical settings. The sensitivity of a biosensor is defined as the ratio between signal generated from the biosensor and the change in

concentration of target analyte. The sensitivity of a biosensor represents the ability of a biosensor to differentiate between different analyte concentrations. The limit of detection (LOD) is defined as the minimum amount of target analyte which can be detected by the biosensor platform. Ideally, a biosensor should have the LOD as low as possible to confirm the presence of the target in biological samples.

The presence of Debye screening effects is one of the main limitations in achieving the ultimate sensitivity and LOD for GFET biosensors. The detection mechanism of the GFET sensors relies on the sensing of the electrical charges of target analytes in the solution. However, interactions between the electrolyte solution and biomolecules at the surface of the sensor can greatly influence its response. The charge of the biomolecules is surrounded by ions in the electrolyte solution, which means that the electrostatic potential of the analyte molecules decays exponentially to approach zero at a certain length. This length is known as the Debye screening length (λ_D). The screening length can be given by, [136]

$$\lambda_D = \sqrt{\frac{\epsilon k_B T}{2N_A q^2 I}}$$

Where ϵ is the dielectric permittivity of the medium, k_B is Boltzmann's constant, T is the temperature, q is the electron charge, and I is the ionic strength of the solution, N_A is the Avogadro's number.

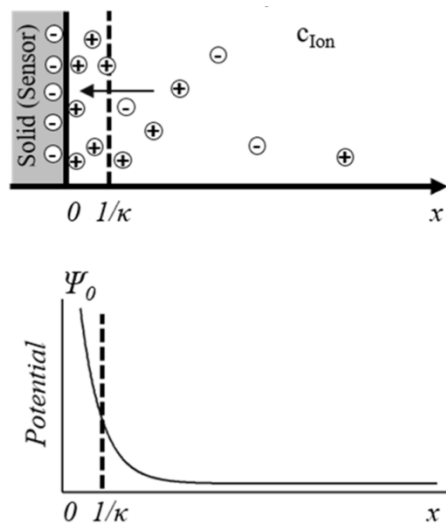


Figure 2-13 Schematic diagram of electric double layer formation and Debye screening at surface. [137]

The Debye length is inversely proportional to the square root of the ionic strength of the electrolyte solution (I). Higher ionic strength solutions will lead to shorter Debye screening lengths. The effective detection of biomolecules requires target binding to occur within the Debye length. For example, the Debye length of 1X phosphate-buffered saline (PBS) solution is around 0.7 nm. This length is suitable for the detection of nucleic acid with a small receptor but inadequate for the detection of proteins that are larger than the screening length. This makes the highly sensitive detection of many types of biological species in real physiological solutions challenging (with Debye lengths <1 nm). The formation of Debye double layer not only limits the detection in high ionic strength solution, but also affects the adsorbed molecules on the surface of graphene. The electrical double layer can affect the orientation and conformation of bioreceptor molecules [86], [138], [139], thereby influencing their functionality and capturing efficiency of target biomolecules. [140], [141] Furthermore, the formation of the electrical double layer structure affects the adsorption of target analytes near the graphene surface. [32], [142], [143] These factors will reduce the capturing efficiency of target analytes, consequently reducing the sensitivity and LOD of graphene biosensor devices.

In addition to sensitive detection, the performance of a FET biosensor relies on its selectivity and specificity in detecting the target biomarkers among other biological species. The specificity of a biosensor is defined as the ability of a sensor to differentiate between target and non-target biomolecules. The performance of a FET is not only determined by the capability of the sensor to sensitively detect biomarkers at the surface, but also to detect them with high selectivity among other biological species. This is particularly important in clinical diagnostics for specific diseases and patient management. Physiological fluids are complex matrices containing many proteins, enzymes, and other biomolecules that can interfere with the graphene biosensors. The non-specific binding of these biomolecules on the graphene surface will lead to false positive signals.

Achieving selectivity in real physiological solutions, such as blood and serum, poses a significant challenge. This is due to the complexity of real physiological solutions and the lower concentration of target biomarkers compared to other biological compounds present in the solution. These non-desired species in the complex biological matrices can adsorb onto the sensor surface, leading to non-specific interactions that lead to false positive or false negative results.

In the application of GFET sensors with clinical solutions, suppression of the background noise is another important factor that needs to be considered. The background noise can arise from multiple sources, including electrochemical noise, environmental interference, and fluctuations from the solution itself. These noise signals can exceed signals from analytes, leading to reduced sensitivity and limit the detection limit. Therefore, improving the noise performance is another important factor in achieving the ultimate sensitivity of graphene-based biosensors and the application of GFET sensors in real physiological solutions.

In addition, the reproducibility and uniformity of lab fabricated GFET biosensors also need to be considered for obtaining reliable sensing results in clinical applications. Batch-to-batch differences in the fabricated GFET biosensors need to be noted and addressed to ensure consistent performance. Meanwhile, the issue of device drift in GFET devices [144], [145], [146] needs to be mitigated for obtaining consistent and reliable sensing performances of GFET biosensors.

It is crucial to address these challenges associated with real clinical physiological solutions and GFET biosensors for achieving the ultimate LoD, sensitivity, and specificity for a reliable biosensor that can be used for early-stage disease detection and disease monitoring.

2.3.2 Strategies of sensing in physiological solutions

Most of the reported GFET biosensors are operated in lab environments with testing in non-physiological solutions. The sensing results from these reported biosensors are difficult to replicate or repeat in clinical settings or with physiological solution conditions. Several techniques have been proposed, aiming to use GFET biosensors for testing in physiological solutions. These techniques will be discussed and introduced in the following sections.

2.3.3 Overcoming the Debye screening effect

The issue with Debye screening can be reduced by operating the GFETs in a diluted buffer with low ionic strength. However, the process eventually becomes difficult and time consuming. It may also reduce the concentration of the target analyte and affect the stability of certain types of biological species. Thus, other strategies have been implemented to overcome the screening effect.

2.3.3.1 Surface Nanostructuring

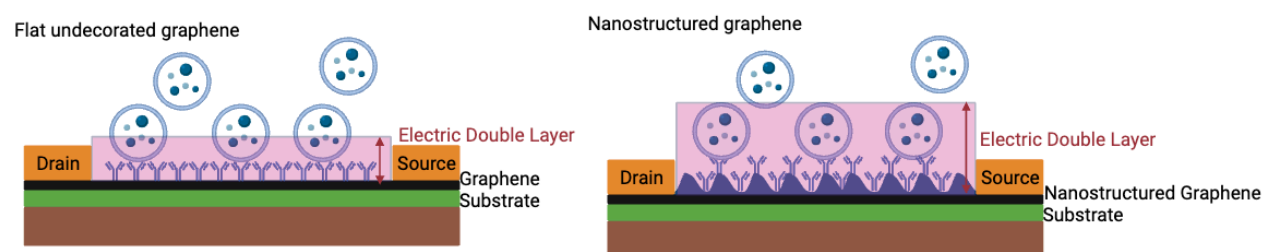


Figure 2-14 Schematics of nanostructured graphene and flat graphene. The use of nanostructured graphene will lead to an increase in the EDL, resulting in an enhanced sensitivity and detection limit of GFET biosensors.

Surface modification of graphene field-effect transistors (GFETs), such as surface nanostructuring, offers a potential solution to overcome the limitation of detection in high ionic strength electrolyte. The application with surface nanostructuring in graphene biosensors is widely reported. [86], [147], [148], [149], [150], [151], [152], [153], [154], [155] Through the fabrication of nanogap and nano-textured electrode [156], [157], the electrode polarization effect can be reduced, leading to a reduced charge screening effect. The effect surface nano-structuring to EDL can be partially explained by the concept of Debye volume introduced by Shoorideh and Chui [158]. Their analytical study of simulated concave and convex electrode surface, as illustrated in Figure 2-15, demonstrated a reduced charge screening effect from concaved surfaces. This effect is attributed to the Debye volume, which

is defined as the volume of electrolyte encompassed within one Debye length from the surface. When comparing an equal sized surface area, the convex surface has a larger Debye volume than concave surface. Therefore, more counter ions present near the surface of the convex electrode surface, resulting in stronger screening effect. Several other experimental studies have demonstrated that modifying the graphene structure or decorating it with nanostructure lead to a lower EDL capacitance compared to planar graphene, resulting in higher sensor sensitivity. These studies demonstrated that the surface nanostructuring can increase the number of accessible sites on sensor surface, leading to higher capturing efficiency and sensitivity of sensor. [86], [147], [149], [151], [152], [154], [155]

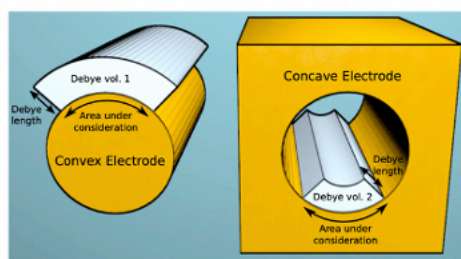


Figure 2-15 Schematic demonstration of Debye volume on a concave and convex electrode surface, for comparison of the Debye volume to surface area ratio. The higher Debye volume indicated stronger charge screening effect (convex surface). [1]

Recently, Ramadan et al. demonstrated the use of carbon dots (CDs) can significantly enhance the sensitivity and detection limit of GFET biosensors for exosome detection. (Figure 2-16 A) [86] This significant improvement of GFET biosensor is based on the combination effect of faster diffusion toward a spherical surface than planar surface [151] and increased capturing efficiencies facilitated by the use of CDs. Kim et al. [151] reported the exponential enhancement of sensitivity and detection limit in graphene biosensors through the use of deformed graphene channels, creating local 'electrical hotspots'. These electrical hotspots enabled the reduction in EDL screening and facilitate a faster diffusion toward sensor surface. They demonstrated the detection limit can be reduced from 2 pM (flat graphene) to 600 zM (crumpled graphene). Hwang et al. [152] further demonstrated the performance of GFET biosensor can be enhanced through increasing the crumpling ratio of the device. (Figure 2-16

B) However, it is worth to note that although the wrinkled improve the sensitivity of graphene biosensors, the surface crumbles can also lead to a reduction in conductivity. Additionally, the effects of graphene wrinkles on biomolecules require further investigation. [159], [160] Through molecular dynamics simulation, Li et al. [159] observed a structural deformation of a double strand DNA segment (dsDNA) when it interacts with a wrinkled graphene surface, while the dsDNA tends to remain its structure upon interaction with idealized graphene sheet. These factors need to be taken into consideration when constructing the GFET biosensors.

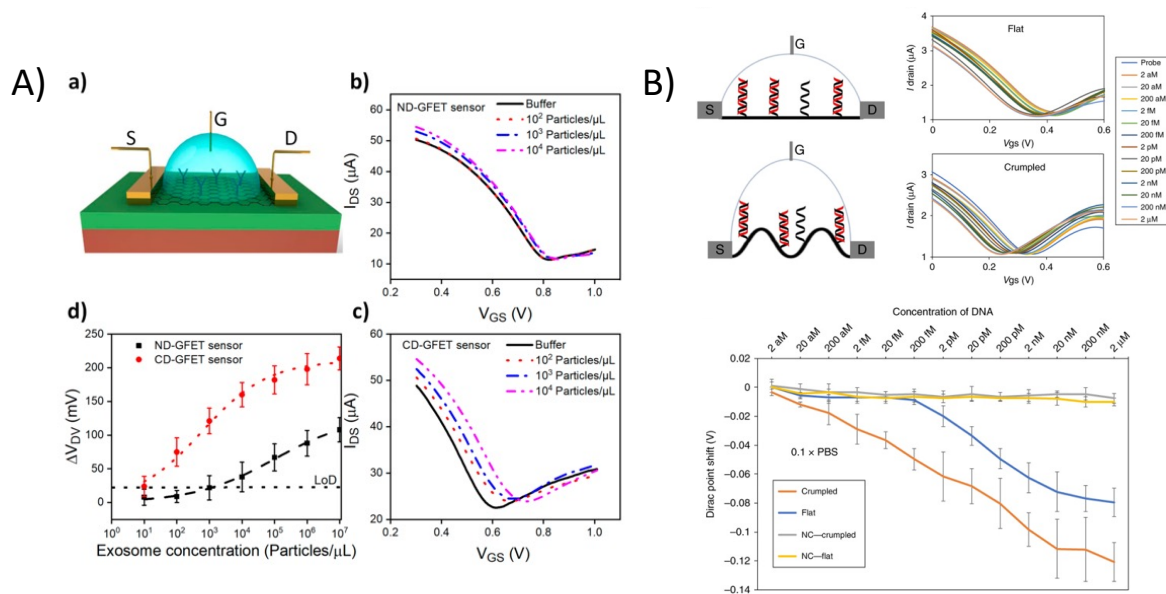


Figure 2-16 Surface nanostructuring for enhancing GFETs biosensor performance. A) Carbon dot decorated graphene field effect transistor. Electrical detection of exosomes on ND-GFET (nondecorated GFET) and CDs-GFET sensors. Dirac voltage shift of GFETs for various concentrations of exosomes on ND-GFET and CDs-GFET sensors.[86] . B) Crumpled graphene Lateral image of the flat (top) and crumpled (bottom) graphene FET DNA sensors. DNA (red strand) is hybridized with probe DNA (black strand) on the graphene surface. I – V relationship of the flat (top) and crumpled (bottom) graphene FET sensors for DNA hybridization. DNA hybridization shifted the I – V curve according to the indicated concentrations. The crumpled graphene's I – V curve shift is significantly more significant than the flat device. [148]

2.3.3.2 Extension of the Debye screening with ion-permeable layers

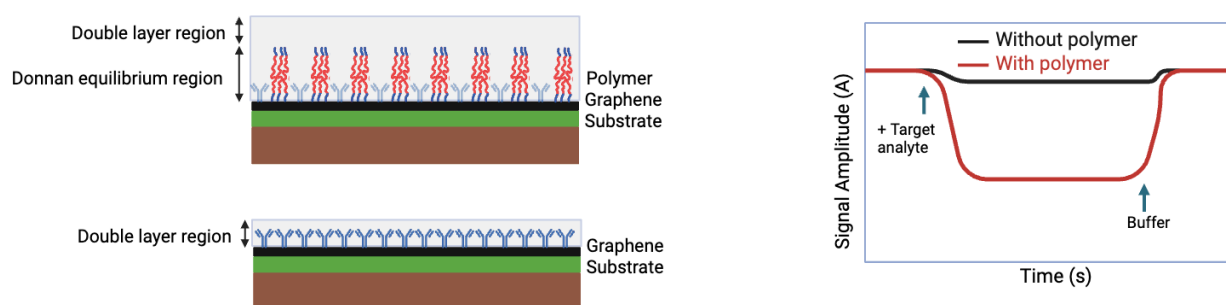


Figure 2-17 Schematics of graphene field effect transistor biosensor with a layer of ion permeable layer. The comparison of GFET device response from time measurements for untreated and polymer coated graphene.

Gao et al. [137], [161] studied the effect of application of a layer of polyethylene glycol (PEG) polymer on the surface of a GFET sensor. They demonstrated that their sensor, immobilized with PSA-specific DNA aptamer and PEG coating, could bind and detect the prostate-specific antigen (PSA) in a high ionic strength buffer solution (1x PBS, pH = 7.4). The signal obtained from their PEG-modified devices was approximately five times larger than that of the unmodified devices. [137], [161] Andoy et al. [162] also demonstrated that through co-immobilization of an antibody fragment and PEG on the surface of a GFET biosensor, their device is capable of detecting protein analytes (thyroid-stimulating hormone) in undiluted serum. The underlying theory behind the effect of this polymer layer is explained by Haustein et al. [163] using an analytical model based on the Donnan potential theory. This theory describes the potential at the interface between the adsorbed ion-permeable layer and the electrolyte as a function of the ions present in the electrolyte. [164] Based on this theory, Haustein et al. [163] found that when PEG is immobilized on the surface, it creates an additional nearly constant potential, leading to an extended Debye screening length. As a result, a higher signal is observed with the presence of a PEG layer compared to the surface without a PEG layer. (Figure 2-18) They further suggested that higher sensitivity of the device can be achieved through immobilization of higher molecular weight PEG. Hu et al. found that fluid-supported lipid bilayer (SLB) floating inside the electrolyte can also detect ligand binding

above the SLB which is outside the Debye length of a solution with physiologically relevant ionic strength [165]. Based on the Donnan potential theory, electrical potential decayed slowly inside the hydrophobic acyl chain region of SLB, resulting in an increase of Debye screening length. However, the device response based on Donnan effect is a combination of Donnan potential and the change in the internal pH induced at the surface. Therefore, achieving a detectable signal from the target biomolecules requires that the sensor to exhibit non-Nernst behavior to prevent that the potential from the Donnan potential and pH shift from cancelling each other. [164]

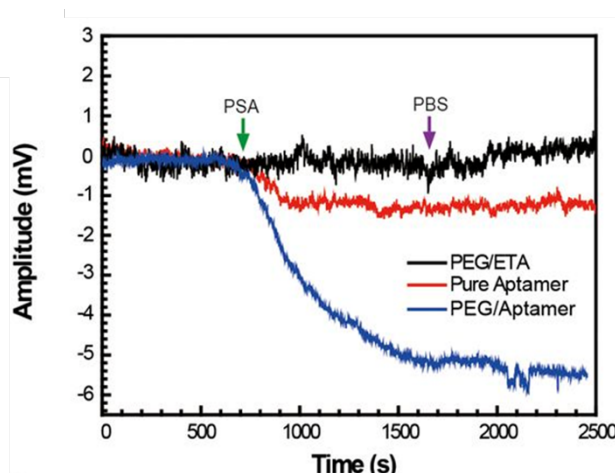


Figure 2-18 GFET device response for detection of PSA in 300 nM in 1X PBS with 1:2 PEG:ETA-, pure aptamer-, and 1:2 PEG:aptamer- functionalized GFET devices. [137]

2.3.3.3 Tailored bioreceptor molecules

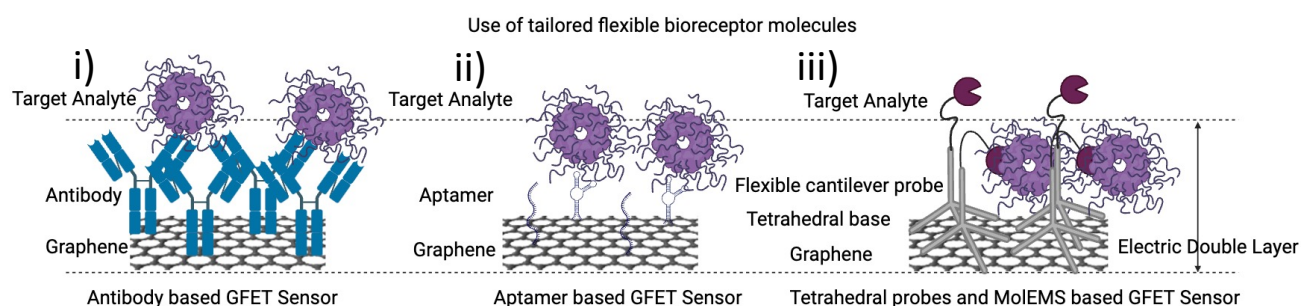


Figure 2-19 Schematics of tailored bioreceptor molecules that can be immobilized on GFET biosensors.

Commonly used bioreceptor molecules, such as antibodies, with sizes up to 10 nm, exceed the Debye screening lengths in physiological solutions. To address this limitation, researchers have explored the use of smaller bioreceptor molecules, such as antibody fragments [162], [166], nanobodies [167], [168], [169], and aptamers [17], [121], [122], [123], [124]. These smaller receptors allow the biorecognition reaction to occur at a closer distance to the sensor surface, enabling improved sensitivity in GFET biosensors. The binding affinity between bioreceptor molecules and target molecules is another critical factor in achieving high sensitivity in GFET biosensors. Aptamers, which are single-strand artificial oligonucleotides, are well-suited for the requirement of high affinity binding and have been widely studied in biosensing applications. [17], [92], [104], [120], [121], [122], [123], [124], [125]. Wu et al. used an aptamer-modified GFET to achieve a sensitive quantification of HepG2 cell-derived microvesicles.[170] This detection achieved a high sensitivity of 84 particles/ μL and selectivity which was able to distinguish the HepG2 microvesicles from other cancer cells. Aptamers offer the advantage of adjustable binding affinity to target molecules by modifying their base sequences, allowing specific binding conformations. Xu et al. [17] demonstrated the superior performance of an aptamer-based GFET biosensor compared to an antibody-functionalized GFET biosensor in the specific and selective detection of SARS-CoV-2 viruses (VLPs) in universal transport medium (UTM). (Figure 2) They achieved an LOD of 160 aM for COVID-neutralizing antibodies in serum. This suggested the potential of aptamers for highly sensitive biosensing applications. Aptamers can bind to the target analytes via various mechanisms, including shape complementarity, electrostatic interactions, hydrogen bonding, and hydrophobic effects. [171] The binding affinity and specificity of aptamers can be further improved through modifications such as introducing chemical modifications of the sequences. Aptamers are produced by the systematic evolution of the ligand by the exponential enrichment process (SELEX) which is more suitable for mass production and large-scale application compared to antibodies.[172] The SELEX process involves multiple rounds of selection and amplification to isolate aptamers with desired binding properties, which is challenging and expensive. Additionally, aptamers are susceptible to degradation by

nucleases in biological media, which can limit their stability and application in biological environments, particularly in blood.[173] In addition to aptamers, other types of tailored bioreceptor molecules have also been investigated to optimize the sensing performance of GFET biosensors, including structural DNA probes and nucleic acid-protein composites [174], [175], [176], [177], [178]. These bioreceptor molecules are designed with distinct structural features that enable precise and selective capture of target molecules on the GFET surface. The tailored bioreceptor molecules can enhance the performance of GFET biosensors. Meanwhile, the production of the synthetic bioreceptor molecules ensured a consistent quality and reproducibility for large-scale production. However, the design and production of these tailored bioreceptor molecules are time-consuming and requires many optimization process to tailor the structure for a specific target. The design and determination of the molecular structure or sequence of specific bioreceptor molecule using computational design is challenging due to the limited availability in databases. [179]

Recently, Wang et al. [177], [178] have demonstrated the use of molecular electro-mechanical system (MoMEMs), a tailored DNA nanostructure with a base and a cantilever, in GFET biosensors. The flexible cantilever part of MoMEMs allows for capturing target analytes in solution. When a negative gate voltage is applied, the cantilever is electromechanically actuated downward, bringing the biorecognition event closer to the graphene surface and overcoming the Debye screening effect. They demonstrated a rapid detection of thrombin and SARS-CoV-2 RNA with LoDs in the range of several copies/molecules in 100 μ l of physiological solution. (Figure 2) Wu et al. [180] further used this tetrahedral probe framework with three probes anchored on top for the detection of SARS-CoV-2 RNA. They showed that the triple-probe TDF dimer GFET sensor can detect SARS-CoV-2 RNA in clinical samples, with a limit of detection of around 1 – 2 copies per 100 μ l of collected clinical samples without the requirement of amplification. Using their method, they demonstrated higher sensitivities, shorter response times and better limits of detection (LoD) of their sensor compared with other established technologies. [180]

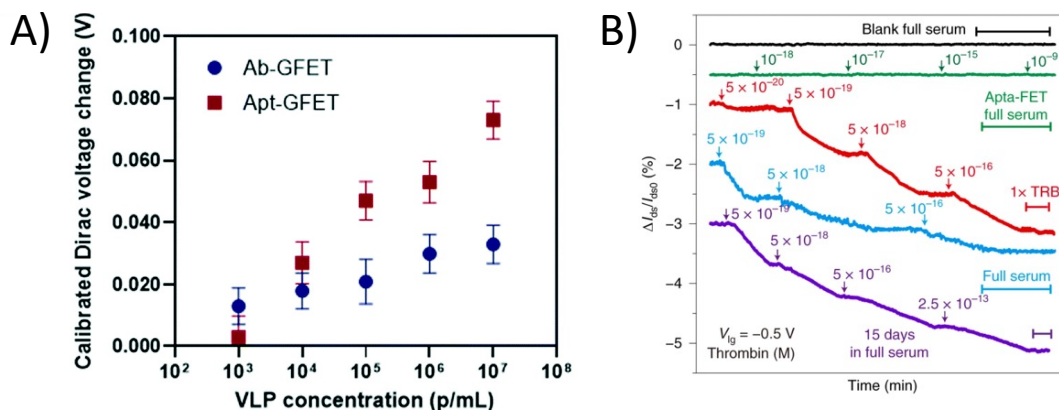


Figure 2-20 Schematics of strategies for overcoming the Debye screening effect using GFET devices with tailored bioreceptor molecules and flexible receptor structures. A) The SARS-CoV-2 detection in universal transport media (UTM) (mimic swab sample) using the on-chip integrated graphene biosensor (Apt-GFET, aptasensor): the comparison results of the on-chip Apt-GFET biosensor (aptasensor) vs. the Ab-GFET biosensor for SARS-CoV-2 virus detection in UTM.[17] B) $\Delta I_{ds}/I_{ds0}$ versus t curves of g-FETs with TBA-functionalized MoEMS in full serum (black) and upon the sequential addition of thrombin in 1x TRB buffer (red) or full serum (blue). The green curve is measured in full serum by a g-FET with a TBA-functionalized 5 T aptamer (Apta-FET). The purple curve corresponds to a g-FET with TBA-functionalized MoEMS after 15 days of incubation in full serum. [178]

The use of gene editing technologies based on clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease (Cas)-based systems has drawn more and more attention [90], [91], [181], [182]. The use of a CRISPR-based GFET sensor is shown to enable the specific detection of a target without the need for amplification. (Figure 2-) Li et al. [90] validated the detection performance of GFET sensors immobilized with CRISPR-Cas13a with the clinical SARS-CoV-2 samples, including those with low viral loads. The Cas13a enzyme mediates the target-triggered trans-cleavage of bystander reporters, allowing for the detection of specific RNA sequences. They also demonstrated the developed sensor can detect the genomes of these viruses down to 1 attomolar without the need for target preamplification.

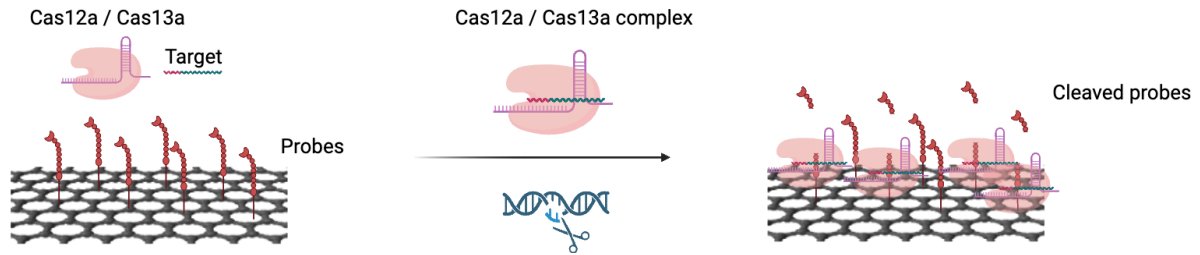


Figure 2-21 Bioreceptor molecules with gene editing technology based on CRISPR-Cas systems.

2.3.3.4 Operating at radio frequency

GFETs operated at high frequency have been proposed as another technique to overcome the Debye screening effects [183], [184], [185]. In electrolyte solutions, ions require a certain time to form the electric double layer at the interface. This time is called τ_c , which is the Debye relaxation time [186]. When FET device is operating at DC or low frequencies, ions have sufficient time to move and screen potentials. When AC or high frequency is applied, the Debye screening effect of the applied potential is believed to be decreased or even canceled out. This is because, at high frequencies, the viscosity of the electrolyte solution causes ions to lag behind the applied field [186]. Consequently, the electrolyte behaves as a pure dielectric at high frequencies, without any charge screening effect. Kulkarni and Zhong [184] demonstrated the possibility of mitigating the ionic screening effect using a heterodyne sensor based on a carbon nanotube-based field effect transistor. In this sensor, FETs are used as high-frequency mixers in a one-source mixing setup. A high-frequency signal is amplitude-modulated with a lower-frequency signal is injected into the source terminal of the FETs. As the molecules are absorbed at the gate of the sensor, the AC voltage causes an oscillating dipole of the bound molecules. This dipole modulation causes conductance change in the transistor channel, which mixes with the AC signal to generate a mixing current. Unlike conventional FETs that is based on charge as the detection mechanism, the heterodyne sensor uses dipoles as the detection mechanism. Using this detection mechanism, the authors demonstrated enhanced sensitivity of their device, and the detection is independent of the ionic strength of the electrolyte solution at high operating frequencies (Figure 2-22). It is

worth noting that the dipole-detection technique in GFET configuration has been further employed to achieve fast and ultra-low detection limits for a range of vapor analytes. The heterodyne sensing technique shows promise as a tool for overcoming the Debye screening effect. However, as the heterodyne sensors operate in the 10 MHz frequency range, it suggests that the ions in the solution can still be affected by the electric field at this frequency. More recently, a X. Zhang et.al [187] has configured GFET biosensor in reflectometry mode at ultrahigh frequencies (UHF) around 2 GHz. At UHF, the ionic screening effect is fully cancelled, and the dielectric sensitivity of the device is enhanced. As the biomolecules bind at the graphene surface, it can change the local capacitance that can varies based on the concentration of the biomolecules. The dielectric-modulated detection using UHF-GFETs has been demonstrated in the real-time monitoring of the binding of streptavidin, and the contractility activities of cardio-myocyte cell. However, the current limitation of the UHF-GFETs biosensor is its complexity in comparison with conventional GFETs biosensor, in addition, further research is required to demonstrate the capability of the device to detect multiple biomarkers.

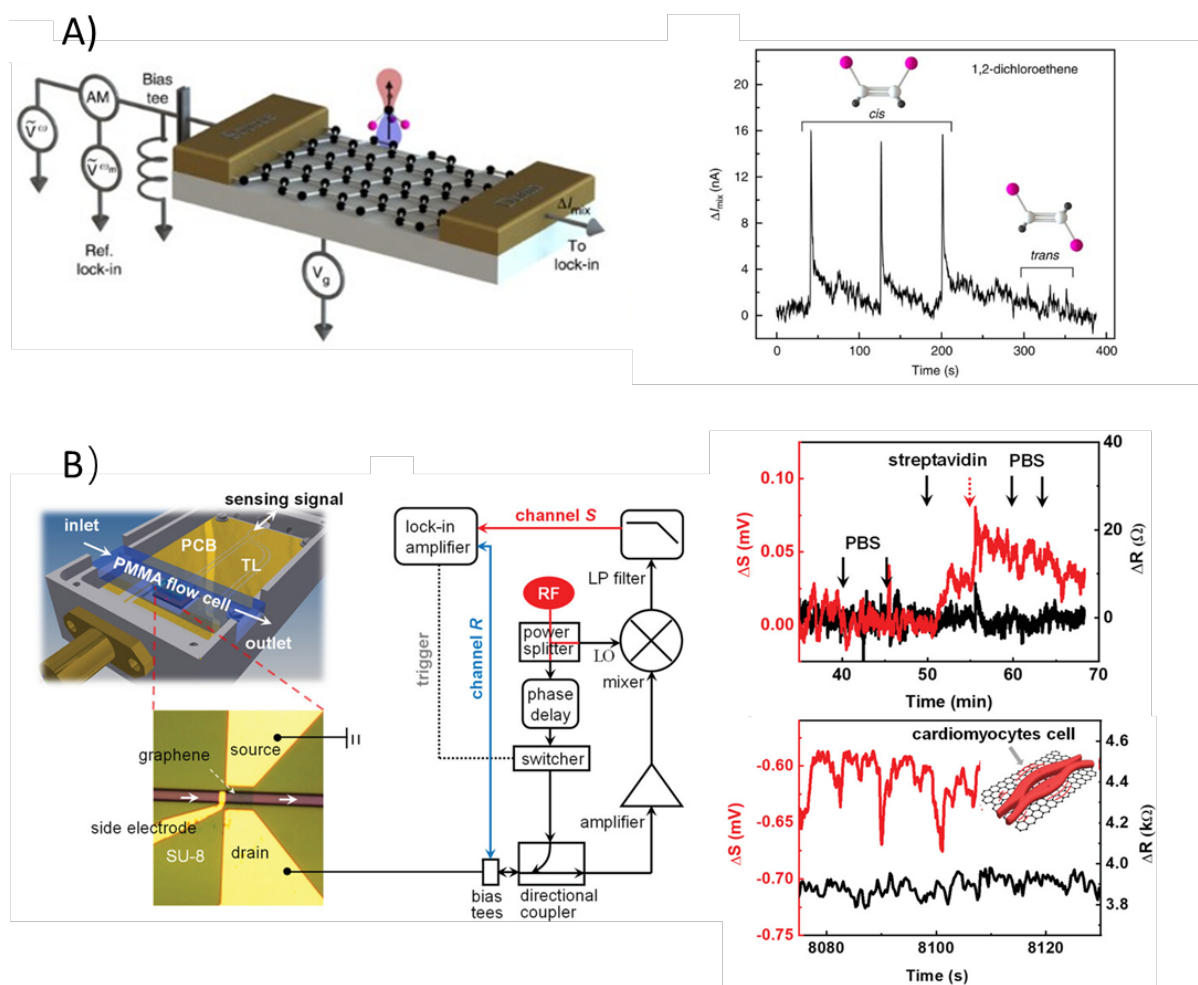


Figure 2-22 Overcoming Debye screening by operating GFETs at radio frequency. **A)** Schematic of GFETs in heterodyne frequency mixing configuration. The interaction of the high frequency (10 MHz) with the biomolecule's dipoles can lead to a change in the mixing current that sensitive to the biomolecule's charges.[188] **B)** GFETs biosensor in reflectometry mode configuration at UHF. The measurements were performed at around 2 GHz. Dielectric modulated UHF signal was demonstrated for the detection of streptavidin and cardiomyocyte cell on graphene. The method shows a detection of the signal beyond Debye screening compared to conventional GFETs. In the figure, dielectric modulation signal is represented in red, while the GFETs signal represented in black. [187]

2.3.4 Strategies to overcome non-specific bindings

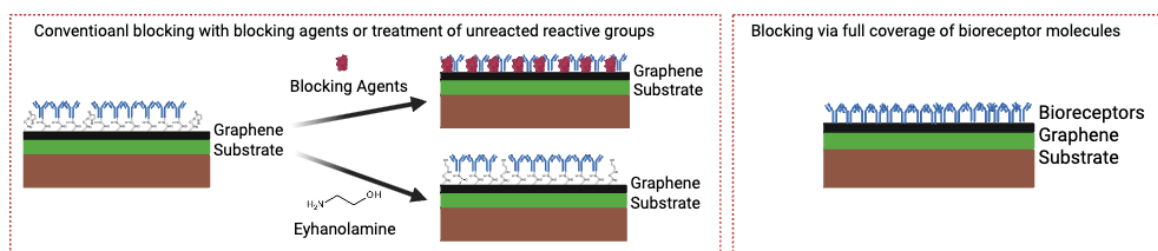


Figure 2-23 Schematics of techniques for overcoming the non-specific bindings. Schematics of various techniques that can be applied, including the conventional methods with blocking agents or the use of surface treatment to react the unreacted reactive groups, blocking via the full coverage of bioreceptor molecules.

Nair and Alam [189] have theoretically demonstrated that the selectivity of a sensor is proportional to the density of surface functionalization. Their findings indicate that high selectivity can be achieved, even in the presence of non-specific binding that is 10^9 times higher than the specific binding, with complete surface coverage. Therefore, surface passivation of the graphene sensor is essential to minimize false positives arising from non-specific binding, especially when detecting blood serum or plasma [190]. Passivation of graphene biosensors can also increase the limit of detection and the signal-to-noise ratio of biosensors by eliminating high background noise signals originating from the unwanted non-specific binding. [190]

There are two primary strategies for passivating graphene-based biosensors and minimizing non-specific binding: (1) the application of a blocking agent and (2) surface treatment. A blocking agent is typically used to fill any uncovered spaces between surface-immobilized bioreceptors. It can also be used to block unreacted reactive groups on linker molecules attached to graphene, ensuring that all binding events arise solely from target-receptor interactions [191], [192]. Commonly used blocking agents include bovine serum albumin (BSA) [192], [193], [194], non-fat dry milk [195], ethanolamine (ETA) [108], [191], [192], [196], [197], [198], polyethylene glycol (PEG) [88], [137], [161], [162], [199], Tween 20 [150], [200], [201], and blocking buffer (BB) [197], [202], [203]. Surface encapsulation of graphene with an ultrathin layer of material, such as Al_2O_3 [204], [205] and propylene [206] is an alternative strategy for

graphene surface passivation. This deposited layer acts as a protective barrier, preventing direct contact between graphene, the electrolyte, and the unwanted species within it, thereby improving the electronic stability of the device. However, this could weaken the electrostatic coupling and impact the GFETs sensor response and its sensitivity. Further it adds extra steps to the fabrication process and increases the complexity of the functionalization process. It is crucial to ensure that the chosen passivation strategy maintains the surface uniformity of graphene without obstructing the binding sites on the bioreceptor molecules. Meanwhile, Wang et al. (83) demonstrated the use of the uniformly immobilized DNA tetrahedral MoEMS structures with high density on the surface of graphene exhibited excellent ability to prevent non-specific interactions. Recently, Yang et al. [207] developed the use of covalent organic frameworks (COFs) with GFET biosensors. A uniform mesoporous crystalline COF film was grown on graphene. They demonstrated the use of COFs as the sensing interface to achieve both detection and prevention of non-specific binding. Although such methods offer high efficiency in blocking the non-specific binding, they also limit the accessibility to the active sites of the bioreceptors, particularly for analytes with larger size. The large analytes may have difficulty reaching the bioreceptors distributed on the channel walls, further affecting the detection efficiency. The thickness of the membrane is usually > 15 nm, which can reduce the sensitivity of the biosensors.

However, non-specific binding can still take place at passivated, blocked, and untreated surface. To minimize their effect on sensor response, a differential measurement can be utilized to reduce the false positives arising from the non-specific binding or sensor drift [17], [20], [92], [94], [125].

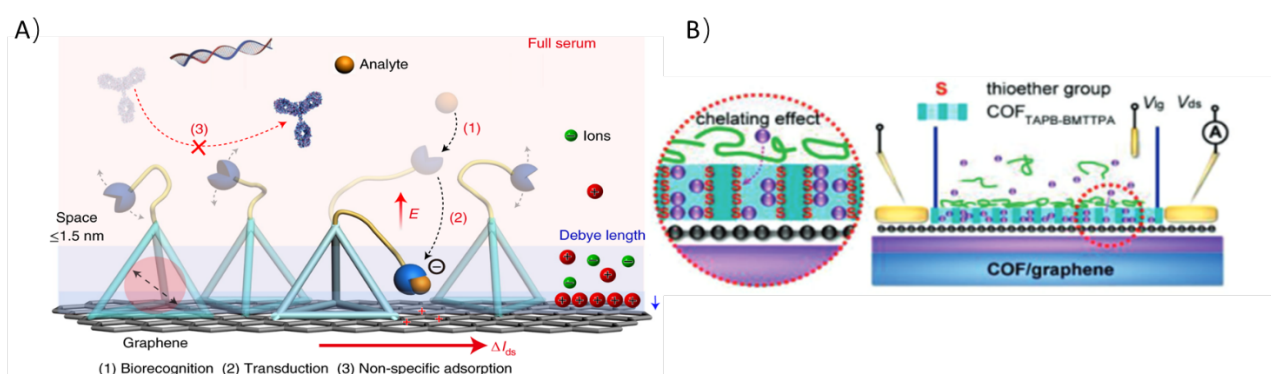


Figure 2-24 Examples of strategies for overcoming the non-specific binding. A) The MoLEMS have high coverage of the surface of graphene, the rigid bases avoid non-specific adsorption.[178] B) Schematic illustration of technique for prevention of non-specific binding by a pristine graphene-based FET sensor and a COF_{TAPB-BMTTPA}/graphene-based FET sensor. The black and red circles show the processes at the sensing interfaces.[207]

2.3.5 Reducing device noise for improved sensitivity

As graphene exhibits extremely large surface where its delocalized electrons can interact with the impurities in the substrate and surrounding surface, this can result in the fluctuations in its carrier concentration, in addition to the mobility fluctuation due to charge scattering. This can lead to conductivity noise fluctuations in graphene devices. High noise levels will result in larger variance in the electrical signal, making it difficult to distinguish between different target concentrations. The high sensitivity of GFETs biosensors is attributed to its ability to response to the small change to the charges near its surface. Therefore, noise fluctuations could significantly hamper the performance of GFETs biosensors. It can be characterized in the frequency domain and at low frequency ($f < 100\text{KHz}$) its spectral density depends inversely on frequency, and it is commonly referred as $1/f$ noise or flicker noise. (130) The existence of $1/f$ noise in GFET biosensors is influenced by complex factors such as surface impurity charge trapping, scattering from the graphene surface, charge puddles, and defects. Both the graphene transistor channel and metal contact can have impact on the $1/f$ noise of graphene transistor devices. [208], [209], [210], [211]

The size of the graphene channel area can be an important factor related to the sensitivity of GFET biosensors. [206], [212] Fakhri et al. demonstrated that maximizing the device's active area can improve the noise performance of the GFET device. The area of the graphene channel is also related to the amount of immobilized bioreceptor molecules and the electronic properties of graphene. A larger active graphene sensing channel area indicates more receptors on the surface, which is closely related to the binding kinetics of target biomolecules. This may lead to enhanced sensitivity and lower the LOD. However, Kireev et al. [212] reported that larger-area graphene devices also exhibit an increased amount of grain boundaries, resulting in reduced charge carrier mobility. They demonstrated that noise performance can be improved by using devices with a smaller graphene channel area.

Currently, there is still no clear conclusion or systematic study on the effect of the graphene channel area size on the sensitivity of GFET biosensors. It is important to consider all the factors that may contribute, including carrier mobility, gate capacitance, underlying substrates, and the active sensing area of GFET biosensors.

The application of biosensors with high-mobility graphene is another preferred option to improve the sensitivity of GFET biosensors [209]. Rehman et al. [213] demonstrated that the fluctuation in mobility of graphene-based electronic devices is closely related to their electronic noise performance. Higher mobility indicates surface with fewer defects, interfacial traps, and charge scattering centers, resulting in lower noise levels of the device. High mobility devices also allow larger sensing responses, leading to better overall sensing performance [206]. Recently, it has been observed that the fluctuation in mobility can be the dominant mechanism of the $1/f$ noise in the graphene devices [213]. Therefore, developing fabrication and transfer methods that can induce low defect density and impurities in graphene is extremely important in achieving high quality graphene with high carrier mobility [32], [142], [143] [208]. The substrate and the dielectric material of the interface can also play a key role in the mobility and noise in the graphene devices. The substrate can act as charge scattering centers due to the long-range Coulomb scattering. Many studies have demonstrated that graphene on hexagonal boron nitride (hBN) substrate can significantly improve mobility in graphene devices. hBN has a small lattice mismatch with graphene and has high dielectric constant that can reduce scattering and screen out charged impurities in the substrate.

Meanwhile, many studies have reported the dependence of the noise level on the gate voltage of graphene. The gate-voltage dependency of the noise level of graphene is found to exhibit a “V” like shape dependence with the minimum shown near its charge neutrality point (CNP) [214], [215]. This behaviour is found to be caused by noise arising from the carrier-number fluctuations, which is induced by the trapping and de-trapping process in the conducting channel through tunnelling. This process is strongly influenced by the location of the Fermi level in the graphene channel, which shifted significantly near the CNP. An “M” shape gate dependence was reported in some devices when larger voltage range was used. This M-shape characteristics was attributed to the disorder in the graphene sheet or the

interplay between long- and short-range scattering. Fu et al. [214] demonstrated that operating the GFET near its charge neutrality point can significantly reduce the $1/f$ noise in graphene with higher signal-to-noise ratio, leading to improved sensing capabilities. They achieved a limit of detection with 2 pM in sensing ssDNA. This indicates the potential of operating GFETs near their neutrality point can improve the sensing performance of GFETs for biosensing applications.

2.4 Applications of GFET biosensor in clinical studies

GFETs biosensors have proven their capabilities of direct detection of various disease biomarkers in real samples, including small molecules, nucleic acids, proteins, viruses, and exosomes. Table 2 summarizes the application and performance of GFETs biosensors for the detection of different disease biomarkers in complex fluidics.

Table 2. Applications of GFET biosensors in clinical studies

Application (Disease)	Clinical samples tested	Detection target	Receptor	Target solution	Limit of Detection (LoD)	Device highlight
N/A	N/A	Nitric oxide (NO)	Hemin	Dulbecco's modified eagle medium (Cell culture medium) or fetal bovine serum or RAW 264.7 macrophage cells or human umbilical vein ECs	1.4 nM in fetal bovine seru; 0.3 nM in PBS	N/A [216]
Cancer diagnosis	N/A	miRNA	Peptide nucleic acid (PNA) probe	Blood serum	10 fM	Use of gold nanoparticles (AuNPs)-decorated graphene field-effect transistor (FET); Use of PNA probe with higher hybridization efficiency and lower background; Spiked miRNA in serum; Incubation of the sample then rinse multiple times

						with PBS and water, measurement performed in PBS. [153]
N/A	N/A	Nitric oxide (NO)	Fe(III) meso-tetra(4-carboxyphenyl)porphyrin (FeTCP) and RGO, named FGPC	Fetal bovine serum (FBS) or Human Umbilical Vein Endothelial Cell Culture	1 pM in phosphate-buffered saline (PBS) and 10 pM in the cell medium	Iron–porphyrin-functionalized graphene (FGPCs) field-effect transistor (FET) biosensor (superior electrical conductivity caused by RGO, and high catalytic specificity induced by metalloporphyrin); 10% fetal bovine serum used; [217] Incubated with cell first, then the cell culture medium removed and measured in PBS.
Cancer diagnosis	N/A	Prostate-specific antigen (PSA)	DNA PSA aptamers	pH 7.4, 1x PBS containing 2 mM Mg ²⁺	N/A	Use of polyethylene glycol (PEG)-modified graphene; Co-modification of graphene devices with PEG and DNA aptamers. [137]
Children blood lead	3	Pb ²⁺ ions	8–17 DNAzyme and the	Blood	37.5 ng/L	Use of 8–17 DNAzyme aptamer;

			Thrombin binding aptamer			Pre-treated blood samples with nitric acid to dissolve cells and proteins and the solution was neutralized by NaOH (1M/L). [120]
Heart failure	3	Brain natriuretic peptide (BNP)	BNP antibody molecule	Whole blood	100 fM	Platinum nanoparticles (PtNPs)-decorated on reduced graphene oxide (rGO); Whole blood was pre-treated with a micro filter system to obtain serum and remove blood cells; Incubation of serum containing BNP followed by rinse. Measurement performed in 0.001×PBS solution. [218]
Zika	N/A	Zika viral non-structural protein 1 (ZIKV NS1)	Zika viral (ZIKV) antigens	1X PBS pH 7.4 or simulated serum	450 pM	Use of C-Response (capacitance of biosensor to the liquid); Use of PEG; Measurement performed at 2 dilutions in quadruplicate of serum. [199]

Hepatocellular carcinoma (HCC)	7 patients	Alpha-fetoprotein (AFP)	Anti-AFP	Blood plasma	12.9 ng/mL	Use of an additional washing step to remove any non-specifically adsorbed HCC patient plasma proteins from the channel surface of GFET; GFET measurements performed in $0.01 \times$ PBS (pH 7.4) [95]
Liver diseases	N/A	Hepatitis B virus (HBV)	Hepatitis B surface antigen-binding aptamer	Diluted blood serum (1%) or artificial saliva	10 aM	Use of multidimensional conductive nanofilm (MCNF), consisting of vertically oriented carboxyl polypyrrole nanowires (CPPyNW) on the graphene layer; Use of HBsAg binding aptamers. [219]
N/A	N/A	Thyroid stimulating hormone (TSH)	anti-TSH F(ab') ₂	Horse blood serum (pH 8)	0.2×10^{-15} M < 0.01 pM	Immobilization of an antibody fragment (F(ab') ₂) and polyethylene glycol on the graphene surface; measured combination of charge neutrality point (CNP) shifts and mobility changes;

						To correct for this chip-to-chip variation, the data were normalized. [162]
Disease diagnosis (Duchenne muscular dystrophy)	6 patients and 3 healthy	CRISPR-associated protein 9 (Cas9) sgRNA	Catalytically deactivated Cas9 (dCas9) CRISPR complex (RNP)	Tissue-derived genomic material from male DMD patients	1.7 fM	Negatively charged DNA target and immobilized dRNPs on the surface of the graphene to create Donnan potential; Use of PEG; Sensor calibration at the first liquid injection before functionalization, use of the percent change in current (I-Response) to remove the effect of variations in resistance sensor-to-sensor; Gate sweeping frequency at 0.3 Hz to mitigate the charge screening effect due to the non-specific adsorption of ions on the surface of the graphene; Incubation of sample then rinse with 2 mM MgCl₂, 30 µL. [183]

Disease diagnosis (Ebola)	N/A	Ebola virus (EBOV)	Equine-origin antibody against the EBOV glycoprotein	Blood serum	2.4 pg/mL	Incubation of sample then rinse with DI water, measurement performed in PBS; Use of EBOV in different concentrations of serum as positive control; Use of different concentrations of blood serum without spiked EBOV as negative control. [94]
Heart failure or COVID-19 diagnosis	N/A	N-terminal pro-brain natriuretic peptide (NT-proBNP) or COVID-19 antibodies	Antibodies	Blood serum	30 pg/mL	150 mM serum buffer. [220]
Cancer diagnosis	N/A	miRNA let-7b sequence	DNA probe	Blood serum	20 aM	Incubation of sample for 1h then rinse with DI; The serum prepared by micro-filtering human plasma;

						GFET incubation overnight with serum before measurement; Blank measurements performed and recorded after stabilization. [148]
Covid-19 diagnosis	3 patients and 2 healthy	SARS-CoV-2 virus	SARS-CoV-2 spike antibody	Clinical nasopharyngeal swab specimens in universal transport medium (UTM)	1.6 × 10 ¹ pfu/mL in culture medium and 2.42 × 10 ² copies/mL in clinical samples	N/A [93, p. 19]
Hepatocellular carcinoma (HCC)	10 patients and 10 healthy	HepG2 cell-derived microvesicles	sulfhydrylated TLS11a aptamer and sulfhydrylated EpCAM aptamer	Blood serum	84 particles/μL	gold nanoparticle (AuNP)-decorated, dual-aptamer modified; MVs were first purified from those blood samples; then incubation of sample followed by rinsing with DI, measurement performed in PBS. [170]
Alzheimer disease biomarker detection	N/A	Amyloid-β (Aβ) and tau protein	Anti-Aβ monoclonal antibody and anti-	Blood plasma and artificial cerebrospinal fluid	21.8 fM (1 pg/mL)	Use of diluted plasma at a ratio of 1:10 in 10 mM PBS buffer (pH 7.4);

			Tau monoclonal antibody			Incubation of sample then rinse multiple times with PBS, measurement performed in 10- μ M PBS; Multiplexed detection using the difference between the pI values of two highly related biomarkers of the disease. [18]
Cancer diagnosis (Colorectal cancer (CRC))	31	SEPT9	Three probes (probe 251, 1031, and 1649)	CRC and corresponding noncancerous tissues	2 ng	Decoration of CDs on GFET; Optimized experimental conditions for DNA sample's dilution and the incubation temperature; Using the combination of three probes; Pre-treatment of samples to get ssDNA fragment suspension. [154]
N/A	N/A	IL-6 and insulin	IL-6 and insulin aptamer	1 $\times$ phosphate buffered saline (PBS) and Diluted Human Urine	618 fM (interleukin-6 (IL-6)) and 766 fM (insulin)	1 mL of urine sample was directly added in 9 mL of PBS at a pH of 7.4; 10 kDa PEG was employed;

						applying electric field at -0.3 V during the PASE immobilization. [88]
Alzheimer disease biomarker detection (neurological diseases)	6 patients and 1 healthy	Glial fibrillary acidic protein (GFAP)	GFAP antibodies	Blood plasma	20 fg/mL (400 aM) in buffer solution and 231 fg/mL (4 fM) in patient plasma	Dilution of patient sample in d1000 PBS 100 times to reduce the viscosity. [193]
COVID-19 diagnosis	20 patients and 10 healthy	SARS-CoV-2 RNA	Phosphorodiamidate morpholino oligos (PMO) probe	Throat swab specimens	0.37 fM in PBS, 2.29 fM in throat swab, and 3.99 fM in serum	AuNP-decorated G-FET nanosensor; Phosphorodiamidate morpholino oligos (PMO) probe (PMO does not have charges, leading to low background signal); Incubation of sample then rinse with DI water, measurement performed in 0.01 × PBS. [175]
Cortisol	1	cortisol	Cortisol aptamer	Saliva	N/A	Use tetrakis(4-carboxyphenyl) porphyrin (TCPP) for biofunctionalization; Use of aptamer. [104]
COVID-19 diagnosis	10 patients	SARS-CoV-2	Single-stranded	Clinical nasopharynge	N/A	Crumpled graphene surfaces;

	and 10 healthy		DNA (ssDNA) RT-LAMP primers	al swab specimens in universal transport medium (UTM)		Sample pre-treatment with dilution and short (10 min) thermal lysis; Use of reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) techniques to achieve RNA amplification. Detection based on primer consumption; GFET incubation overnight with 0.1 x PBS before measurement; Measurement performed and recorded after stabilization; Incubation of sample then rinse multiple times with PBS, measurement performed in PBS. [155]
COVID-19 diagnosis	60	SARS-CoV-2	Multiantibodies (CR3022, n3021 and S1)	Clinical nasopharyngeal swab specimens in universal transport medium (UTM)	3.5×10^{-17} g/mL	Use of multiantibody approach for enhancing antigen-binding affinity. [89]

COVID-19 diagnosis	N/A	IFN- γ , TNF- α and IL-6	IFN- γ specific aptamer, TNF- α aptamer and IL-6 aptamer	Blood serum, saliva and urine	476×10^{-15} M (IFN- γ), 608×10^{-15} M (TNF- α), or 611×10^{-15} M (IL-6)	Use of aptamers; Passivation with Tween 80; Sensor response signal is calculated on the basis of the differential value between the signal values acquired respectively from the sensing channel and the reference channel. [125]
Cancer diagnosis (Breast Cancer)	12 patients and 12 healthy	Breast cancer markers miRNA (miR-4732, miR-191, miR-21, and miR-125)	DNA probes	Blood serum	1 fM	Use of poly-L-lysine (PLL)-functionalized graphene field-effect transistor (PGFET) biosensor (DNA probe density enhancement promoted by the strong electrostatic force of PLL in the channel of PGFET biosensor); Incubation of sample followed by rinse; [221] Optimized incubation time between DNA probe in the channel of the PGFET biosensor and DNA-miRNA complex.
COVID-19 diagnosis	N/A	Spiked SARS-CoV-2 RNA		Throat swab specimens		

COVID-19 diagnosis	18 patients and 7 healthy	SARS-CoV-2	Angiotensin converting enzyme 2 (ACE2)-Fragment crystallizable (Fc) chimera	Clinical nasopharyngeal swab specimens	20 pg/mL (recombinant Spike protein) and 65 cps/mL (swab samples of SARS-CoV-2)	Use of ACE2-Fc chimera for improved stability and affinity for viral detection in functionalised gFET; Incubation of sample then rinse with 10mL PBS, measurement performed in PBS. [166]
COVID-19 diagnosis	9 patients and 5 healthy	SARS-CoV-2	CRISPR Cas13a; RNA reporter (PolyU ₂₀)	Clinical nasopharyngeal swab specimens in universal transport medium (UTM)	1 aM	CRISPR Cas13a-gFET relies on the Cas13a-mediated RNA trans-cleavage on gFET for sensor signal transduction (cleavage-based CRISPR diagnostics; Optimization of CRISPR Cas13a-gFET conditions; Use of PEG; Use of multiple parallel detections with an array of six GFETs on wafer. [90]
COVID-19 diagnosis	16 patients and 20 healthy	SARS-COV-2	DNA aptamer	Throat swab specimens	1.28 plaque-forming units (PFU)/mL (S protein) and	Use of aptamer; Quality control metric for GFET fabrication; quality control measures were

					1.45 PFU/mL (N protein)	adopted;(exclude GFET devices, data rejection criteria applied); Use of internal control (data with aptamer alone as a baseline control); Stabilization of GFET with 0.1× PBS (or saline) for 30 min prior to introduction of sample; Incubation of sample then rinse multiple times with PBS, measurement performed in PBS. [92]
COVID-19 diagnosis	N/A	SARS-CoV-2	Neutralizing antibodies	mixture solution of 0.05× PBS and artificial saliva	a few pg/mL	Remote floating-gate (RFG) FET configuration; Measurement after device stable; Controlling pH of testing media at pH7.4 via mixture of saliva sample with PBS. [222]
COVID-19 diagnosis	N/A	SARS-CoV-2 virus-like-	Aptamer	Universal transport medium (UTM)	10 ³ particles/mL (virus-like particles (VLPs)) in clinical	Use of on-chip independent internal control channels for

		particles (VLPs);			transport medium and 160 aM (COVID-19 neutralizing antibodies) in serum	background signal measurement; Use of aptamers. [223]
COVID 19 diagnosis	33 patients and 54 healthy	SARS-CoV-2 RNA	Aptamer	Clinical nasopharyngeal swab specimens in universal transport medium (UTM)	one to two copies in 100 µL	Use of molecular electromechanical system (MoEMS) consisting of an aptamer probe bound to a flexible single-stranded DNA (ss-DNA) cantilever linked to a self-assembled stiff tetrahedral double-stranded DNA (ds-DNA) structure; the MoEMS g-FET devices were immersed in UTM for 1-2 h before use. [178]
Diseases diagnosis (Myocardial injury)	15 patients	Cardiac troponin I (cTnI)	Single-stranded nucleic acid cTnI azide-aptamer with TTT-TCA loop	Blood serum	3.34 pg/mL	4-((triisopropylsilyl)ethynyl)benzene diazonium salt (TIPS-Eth-ArN ₂ ⁺) functionalized graphene, use of “click” chemistry

			and a CCCTC portion			aptamer-modified graphene-based FET (GFET); polyethylene glycol (PEG) modified; measurement performed and recorded after 10 min stabilization for each sample; Sample pre-treatment with desalination columns prior to GFET sensing. [61]
DNA detection	N/A	single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA)	CRISPR Cas12a	2 mM MgCl ₂ or 1× PBS	1 aM (ssDNA human papillomavirus 16 synthetic target) and 10 aM (dsDNA <i>Escherichia coli</i> plasmid target)	Cas12a-mediated multiturnover collateral cleavage of nonspecific DNA probes for signal amplification; Use of an array of 48 GFET channels on sensor; Soak the graphene channels with the test solution for stable signal responses and then rinsed with the test solution and water prior to the measurement. [91]

COVID-19 diagnosis	N/A	SARS-CoV-2 antigen and RNA	Protein catalyzed capture agents (PCCs)	10% saliva spiked sample in 1X PBS	10^3 in buffer and 10^4 PFU/mL in 10% saliva, ≈ 65 am for viral RNA isolate	Unique protein catalyzed capture bioreceptors (PCCs) for antigen capture and a chimeric (RNA-DNA) probe for RNA detection; analysis of charge neutrality point (Dirac point) and relative I- and C-responses (%); the role of internal and external factors with respect to gate voltage is further analyzed by Principal Component Analysis (PCA); Incubation of sample then rinse multiple times with PBS, before measurements. [87]
--------------------	-----	----------------------------	---	------------------------------------	---	--

2.5 Summary

Graphene field-effect transistor (GFET) biosensors have been demonstrated the capability for ultrasensitive, selective, multiplexed, and rapid detection of disease biomolecules. Moreover, the ability to integrate their fabrication with standard semiconductor manufacturing technology and portable devices could enable their application in point-of-care (POC) diagnosis. However, there are still several challenges to overcome and evaluation tests to be established before the clinical translation of GFET biosensors into POC diagnostic devices. First, the detection of disease biomolecules in real samples such as blood or serum requires the use of graphene biosensors that can function effectively in these complex environments. These fluidic materials have extremely low Debye lengths, which restricts the electrical response of the sensor and consequently the detection limits. Methods such as surface nanostructuring, flexible receptor structures, and ion-permeable films have been developed to enable detection in high ionic strength environments. However, these methods still need to be demonstrated for repeatable detection in clinical samples for different types of biomarkers. Additionally, while these methods can extend the Debye screening length by a factor of two or bring the target to the surface of the transducer, they may be more practical for the detection of small biomarkers such as nucleic acids or small proteins. The detection of larger biomarkers such as viruses or exosomes can benefit from these methods, but their high-efficiency detection in blood samples is not as efficient as most of the biomarkers will be outside the screening length. So far, the most suitable way to detect large biomarkers such as exosomes and viruses is to perform the testing in a dilute buffer solution. However, this process is time-consuming and can affect the structural conformation of the receptor/analyte complex. Secondly, although graphene sensors are capable of multiplex detection, which is beneficial for the reliable diagnosis of diseases such as cancer, there is a need for the development of robust protocols to eliminate cross-talk and non-specific binding effects. These effects can increase the complexity of device manufacture and cost, as well as the time required for analysis. Thirdly, the translation of GFET technology into POC devices requires validation with a large number of various types of clinical samples. Although many researchers have demonstrated GFETs for the detection of disease biomarkers in real samples, their work was more as a proof-of-concept of the technology in the clinical setup. More samples are required to test the specificity and reliability of the detection.

Another important requirement for technology transfer to POC diagnostic devices is to establish robust criteria for sensor manufacturing and test results. Device-to-device and chip-to-chip uniformities, in addition to the quality of graphene and standard manufacturing and functionalization processes, are critically important not only to achieve reproducible results but also in sensor performance. Similarly, the detection environment, test protocol, and analysis of test results must be critically considered in clinical testing.

Although research into graphene biosensors has reached an advanced level of development, including the detection of disease biomarkers with high performance in real-time, and relatively low-cost device fabrication with miniaturization and integration capability, the implementation of this technology into future health applications requires overcoming the outstanding challenges regarding the improvement of the detection in highly complex biological environments. Additionally, further efforts must be devoted to standardizing the manufacturing and testing protocols, in addition to further development needed in the electronics and data processing. The successful resolution of these challenges could pave the way for the adoption of GFET biosensors in POC diagnostics, with the potential to revolutionize the way that diseases are diagnosed and monitored.

3 Materials and Methods

3.1 Graphene Transfer

The monolayer of graphene grown by chemical vapour deposition on (111) copper foil was purchased from Graphene Supermarket, Graphenea and GrollTex. To fabricate GFET devices, the graphene was removed from the copper substrate and transferred onto selected substrates. Wet graphene transfer process was employed. The detailed summarized process is shown in Figure 3-1.

Firstly, graphene was cut into the desired size and spin coated with a layer of 6 % poly (methylemethacrylate) (PMMA) in anisole (obtained from MicroChem Corp). A vacuum spin coater (MTI corporation) operated at 5000 rpm for 10 seconds followed by 7700 rpm for 30 seconds is used. This spin coating process is performed to protect and act as a support for the monolayer graphene during the transfer process. The samples were then kept at room temperature for curing overnight.

Secondly, oxygen plasma was performed with a Sentech Etchlab 200 Reactive Ion Etching machine for 1 minute at power 75 W to remove the graphene at the backside of Cu foil. Alternatively, 10% nitric acids can be used to clean the backside graphene. Next, ammonium persulphate ($(\text{NH}_4)_2\text{S}_2\text{O}_8$ (APS) (purchased from Sigma-Alrich) is used to etch away the copper foil. APS was first dissolved in de-ionized (DI) water to a concentration of 0.01 g/mL for making the APS solution. Then, PMMA/graphene/Cu sample was gently placed on the solution for overnight etching. Once the backside copper is etched, the PMMA/graphene sample is then gently transferred for two consecutive ultra-DI water baths to rinse the graphene surface from residues for 15 – 30 minutes per bath.

Next, the PMMA/graphene was gently transferred onto modified RCA1 solution, made up with 20:1:1 $\text{H}_2\text{O}/\text{H}_2\text{O}_2/\text{NH}_4\text{OH}$, for 0.5 hours at room temperature followed by two further steps of ultra-DI water baths for a minimum of 0.5 hour each. This removed the Cu residues and residual APS ions under the graphene.

The floated free-standing PMMA/graphene was then transferred onto the chosen substrate, either SiO_2/Si or PTFE/ SiO_2/Si substrate. The SiO_2/Si substrates are pre-cut SiO_2/Si substrates

with a size of 1 x 1 cm² (purchased from Crystec GmbH) or uncut wafers (purchased from Graphene supermarket). The uncut wafers were cut into the desired size using a diamond scribe. All substrates were cleaned using the following protocol before use: sonication in acetone for 10 minutes, followed by sonication in isopropanol for 5 minutes and sonication in DI water for 2 minutes for three times. All cleaned substrates are then dried with N₂. The PMMA/graphene was then transferred onto the selected substrate. A dental mirror was used to help the transfer process, with tape on the dental mirror to attach the substrates. The dental mirror with cleaned substrates was first carefully submerged underneath the floated graphene and lifted carefully until the substrate came into contact with the graphene. The PMMA/graphene was then adhered to the surface of the substrate. PMMA/graphene/substrate was left to dry overnight at room temperature. The PMMA/graphene/substrate sample was baked on a hotplate at 180 °C for 1-2 hours to ensure the water was completely removed and to improve the adhesion between graphene and the substrate.

The PMMA was then removed using 5 hours of acetone followed by 5 minutes of isopropanol and 5 minutes of DI water. Then the samples were annealed at 200 °C for 6 hours to remove the residual PMMA on the surface of graphene. The transferred graphene on the selected substrate can then be further used for characterization and device fabrication.

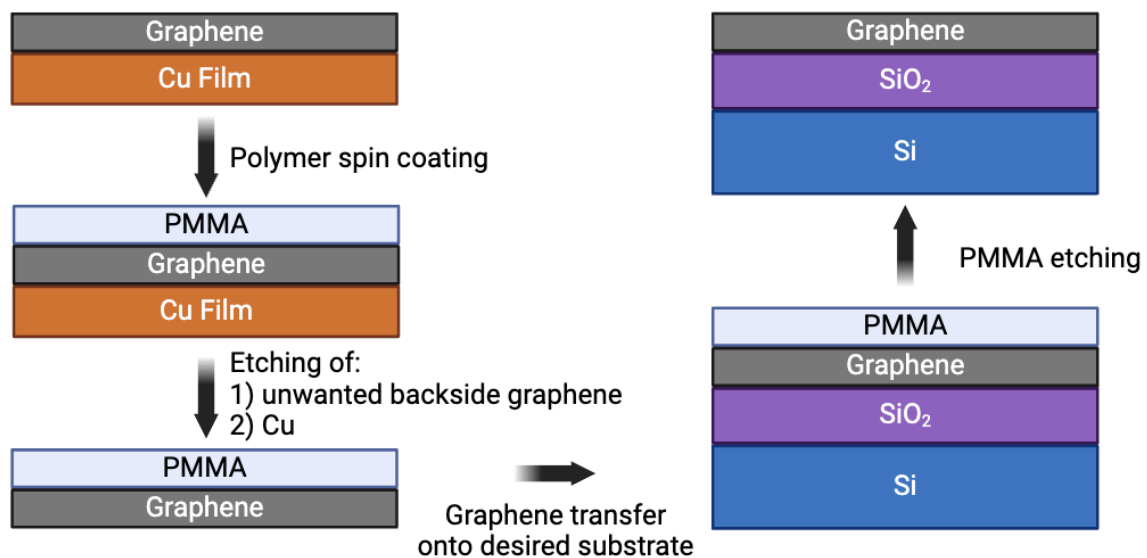


Figure 3-1 Schematic diagram of the wet graphene transfer process. The wet graphene transfer process is used to transfer the CVD graphene on copper onto the desired substrate.

3.2 Characterisation Methods

Multiple physical analysis and chemical analysis with different characterisation methods were applied to investigate the quality and properties of graphene. The aim of this section is to provide an overview and introduction of the working principles of the characterisation techniques used in this thesis. All methods are described below.

3.2.1 Optical Microscopy

The optical microscopy imaging is a simple and useful tool for a preliminary investigation of the quality of graphene. It provides the optical contrast with differences in intensity and colour that allow detailed features of the specimen to become visible. It is a non-destructive technique that can provide information about the structural crystallinity properties of graphene. The optical microscopy can provide information about the number of layers, the existence of scratches on the graphene and the impurities presented.

Due to the differences in refractive index between graphene and its underlying substrate, the colour contrast allows identification of the graphene and the substrate. The number of layers of graphene can also be easily identified using the optical contrast. Multiple-layers of graphene appear darker than single layer graphene under the optical microscope. Impurities present in graphene can also be easily identified using different colours of spots presented as the incident light gets reflected differently.

Optical microscopy was performed with Olympus System BX51 compound optical microscope, as shown in Figure 3-2. A range of objective lenses were used, from 1.25 to 100x magnification.



Figure 3-2 Olympus System BX51 compound optical microscope. [224]

3.2.2 Atomic Force Microscopy (AFM)

Atomic Force Microscopy (AFM) is a powerful technique that characterizes the graphene surfaces and its nanoscale structures with exceptional resolutions. It is one type of scanning probe microscopy with Angstrom resolution. AFM can be used to characterize graphene samples without the need of complexed sample pre-treatment, it can be operated under ambient conditions or even physiological conditions.

AFM can provide information including surface roughness, surface topographical changes and surface morphologies. It is especially useful in providing information when graphene is functionalized with different molecules or nanoparticles. AFM allows the identification of the number of layers of graphene, the quality of graphene after functionalisation, and the distribution of biomolecules and their morphologies on graphene surfaces.

There are different modes of AFM, including the standard scanning AFM modes and the advanced imaging modes. The standard scanning AFM modes include contact mode, tapping mode and non-contact mode. The contact mode of AFM is the simplest operational mode of the AFM. During the scanning of the sample, the AFM probe is in continuous contact with the sample surface. The detector monitors the deflection of the cantilever and calculates the force using Hooke's law. In the tapping mode, the cantilever oscillates at or slightly below its resonance frequency. The feedback system in the AFM maintains the cantilever at a constant

amplitude. In the non-contact mode, the cantilever oscillates at slightly above its resonance frequency. The cantilever oscillates near the surface of the sample, while not contacting it.

There are also several advanced imaging modes available, including the conductive AFM, magnetic force microscopy (MFM), etc.. The magnetic force microscopy (MFM) depends on the detection of magnetic fields by using a magnetic probe in the AFM. The MFM is widely used for characterizing samples that are magnetic materials or samples with magnetic properties. The conductive AFM is used to characterise the conductivity of the examined sample and its electrical properties, including its nanoscale charge transport and charge distribution. The conductive AFM can also provide information about the dopant profiles of the semiconductors. The application of conductive AFM is achieved with a sharp conductive probe.

The basic set-up and main components of an AFM are shown in Figure 3-3. A sharp tip is placed at the end of the AFM cantilever that can scan over the surface of the sample. The motion of the cantilever is achieved by a small piezo element in the cantilever holder. Silicon nitride cantilever is used for all measurements. For measurements with non-contact mode or tapping mode, which is the mode used in this thesis, the AFM tip oscillates close to the surface of the sample without making any contact. Tapping mode is selected due to its advantageous in minimizing sample damage or distortion while achieving high lateral resolution, making it suitable for the compositional and topographical mapping of biological samples immobilized on the graphene surfaces. During the operation with tapping mode, the frequency and amplitude of the cantilever are kept constant at or near its resonance frequency. When the tip comes close to the surface through van der Waals forces, electrostatic forces, etc., the amplitude of the oscillation of the cantilever changes accordingly. This amplitude is then fed into a feedback loop that allows the adjustment of the height of the cantilever above the sample. The change in the height of the cantilever allows the construction of the image of the sample.

All AFM was performed using Asylum MFP-3D classic and a Bruker Innova system in AC tapping mode with SCOUT 70 tips of average radius 15 nm and typical scan resolution of 512 pixels × 512 pixels. Topography AFM images were recorded. All AFM scans were performed in dry conditions.

For each sample, several AFM micrographs were taken at different areas. For the quantitative analysis of each sample, 3 to 5 images were recorded and processed using Gwyddion image analysis software.[225] Images obtained were analyzed using ImageJ [226] to obtain the density of particles on the graphene surface. Images were processed using a colour threshold with the threshold colour set as B&W and colour space as RGB, with the same threshold value applied. Then, the particles were counted using the “analyze particles” toolbox in ImageJ.

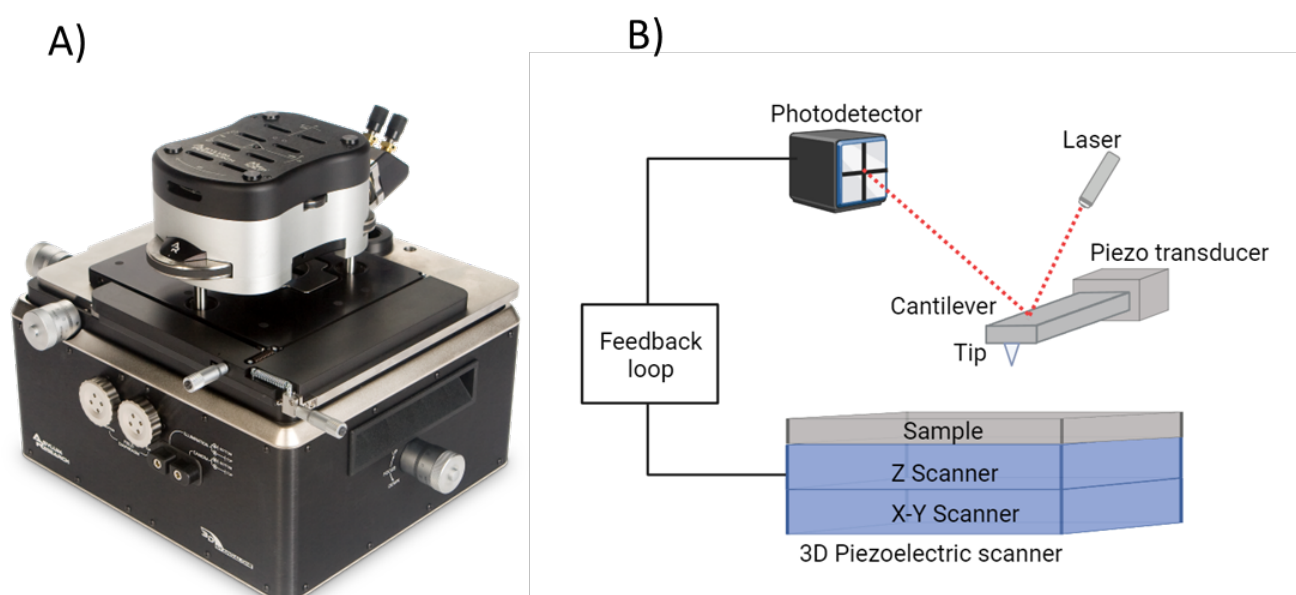


Figure 3-3 Atomic force microscopy. A) Asylum MFP-3D classic atomic force microscope. B) The primary components of an atomic force microscope, including a 3D piezoelectric scanner with Z Scanner Electronics and X-Y Scanner Electronics, a laser, cantilever, and photodetector.

3.2.3 Fluorescent Microscopy

Fluorescent microscopy is a powerful and useful technique for imaging samples with specific features. This is accomplished by attaching fluorophores or fluorescent molecules to target features, which can absorb light at one wavelength and emit a longer wavelength light. This emitted fluorescent light upon excitation makes fluorescent microscopy particularly useful in characterizing the expression and distribution of specific biomolecules on the surface of

graphene biosensors. This information can help in advancing the knowledge of the quality of graphene surface functionalization.

The configuration of a confocal fluorescence microscopy is illustrated in Figure 3-4. Through labelling the functional groups or biomolecules (e.g., antibodies, probes, exosomes) with fluorescent probes, they can generate fluorescent signals. The emitted fluorescent signals can be separated from the surrounding radiation and the illumination light through the spectral filters. This allows only specific features on the surface of graphene with fluorescent labelling to be seen. The fluorescent imaging provides valuable insights into the graphene sensor's sensitivity, specificity, and spatial distribution of surface binding events. This information is crucial for the development and optimization of graphene biosensors.

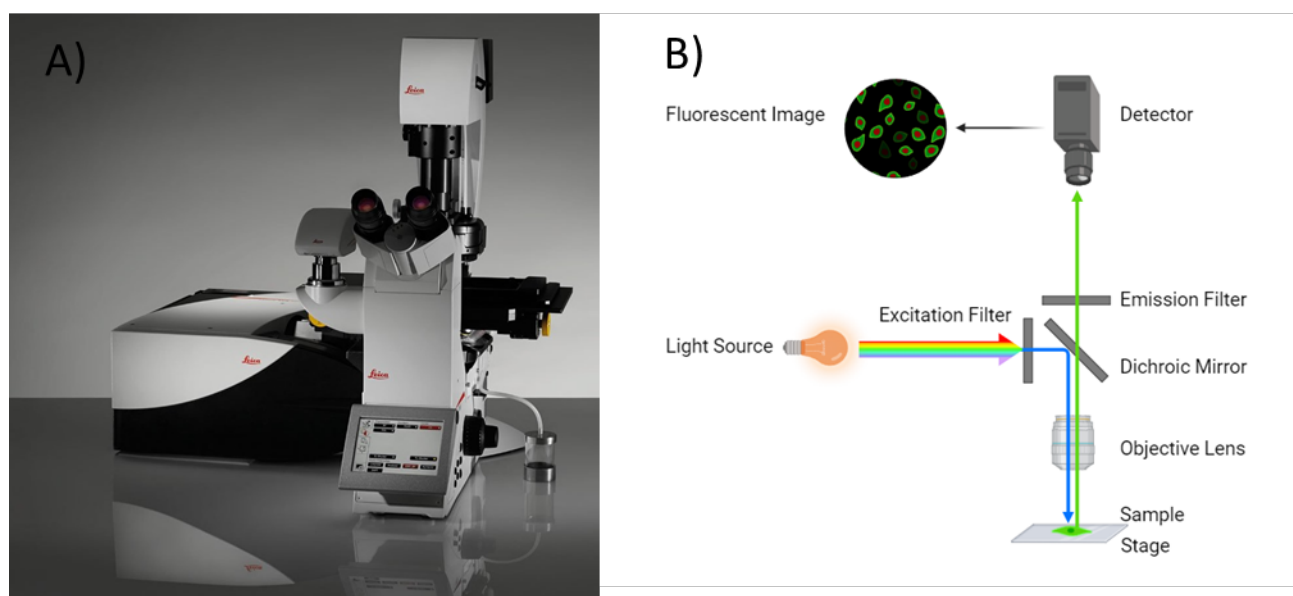


Figure 3-4 Fluorescent microscope. A) A Leica SP8 point scanning microscope. B) The primary components of a fluorescent microscope.

For the characterization of antigen binding efficiency to surface-immobilized antibodies on different substrates functionalized with different linker molecules, the detailed method is described below.

Sample immunofluorescent labelling: To obtain the antigen binding efficiency to surface-immobilized antibodies on different substrates after surface functionalization with TCPP or PBASE, the sample surface was conjugated with 5 μL of 0.5 mg/mL of anti-CD63 antibodies

(BD Biosciences, #556019) overnight at 4°C in a humidified environment. After antibody conjugation, the samples were rinsed with 1 × PBS and DI water and dried with N₂. The surface was then blocked using 1% bovine serum albumin (BSA) in 1 × PBS at room temperature for 1 h, then rinsed with PBS and DI water, and dried with N₂. Next, the samples were incubated with a 10 µg/mL fluorescent exosome solution (Novus, #NBP3-11696) in phosphate buffer saline for 15 min at room temperature in the dark. The samples were then rinsed with 1000 × PBS and DI water and dried with N₂.

For confocal microscopy, surfaces were placed upside-down in a 35mm Ibidi imaging dish and imaged on a Leica Stellaris 5-point scanning microscope with a HC CS2 Plan Apo 63x/1.4NA objective, with a zoom of 4 and 1024x1024 pixels (px) per frame, giving a xy pixel size of 45 nm. Exosomes were labelled with fluorophores (excitation maximum of 500 nm – 650 nm and emission maximum 510 – 665 nm) and excited at 485nm with a white light laser. Fluorescence was detected on a HyDS detector between 496 and 653 nm. 16bit LIF files were opened in Icy[227] and exosomes were detected using the Spot Detector plugin [228] in with a minimum size of 70px.

For the comparison of expression of cancerous PDAC exosomes and healthy exosomes on the surface of graphene immobilized with GPC-1 antibody, the detailed method is described below.

Sample immunofluorescent labelling: After surface functionalization with TCPP and activation with EDC/NHS at room temperature, the sample surface was then conjugated with 5 µL of 0.33 mg/mL of anti-GPC-1 antibodies (Invitrogen PA5-28055) overnight at 4°C in a humidified environment. After antibody conjugation, the samples were rinsed with 1 × PBS and DI water, and dried with N₂. The surface was then blocked using 1% bovine serum albumin (BSA) in 1 × PBS at room temperature for 1 h, then rinsed with PBS and DI water, and dried with N₂. Next, 20 µL of blood plasma samples were added to the surface and incubated for 0.5 h. The samples were then rinsed and labelled with Alexa Fluor 647 anti-GPC-1 antibodies (Abcam, ab237290) (20 µL, 1:100 diluted in 1 × PBS) at room temperature for 1 h in the dark. The samples were then rinsed with 1000 × PBS and DI water, and dried with N₂. For confocal microscopy, surfaces were placed upside-down in a 35 mm Ibidi imaging dish and imaged on a Leica SP8 point scanning microscope with a 63x/1.4NA Plan Apo objective, with a zoom of

4 and 1024×1024 pixels (px) per frame, giving a xy pixel size of 45 nm. Exosomes were labelled with Alexa Fluor 647 anti-GPC-1 antibodies (Abcam, ab237290) and excited with a 633 nm laser. Fluorescence was detected on a HyD detector between 643 and 782 nm.

16 bit LIF files were opened in Icy[227] and exosomes were detected using the Spot Detector plugin,[228] with a minimum size of 10 px. A rectangular ROI of 240 px by 120 px was drawn in the field of view in a region without detection, and the mean fluorescence intensity was calculated. Only detection cases with a mean fluorescence intensity of at least twice the background intensity were counted.

3.2.4 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is a versatile and high-resolution imaging technique that is widely used to study surface morphology, surface topography and surface elemental information. SEM employs a focused beam of electrons that scans across the surface of the sample. These high-energy electrons interact with the atoms on the sample's surface and generate various signals, including secondary electrons (SE), backscattered electrons (BSE), X-rays, Auger electrons and photons of various energies. These signals are collected and used to produce images of the sample's topography and composition. The magnification level that SEM can achieve is at the nanometer scale according to the sample and selected system, offering a great opportunity for characterising nanofeatures on the surface of graphene. It is worth to note that the samples that can be characterised using SEM need to be either conductive or coated with a conductive layer.

However, there are some difficulties associated with SEM imaging of graphene and biological species on graphene. First, the thin layer of graphene is too smooth for the SEM to generate images with sufficient contrast. Second, due to the resolution limitation of SEM for imaging the atomic thin layer of graphene. The SEM image may display the morphologies of the substrate underneath the graphene. Thirdly, the SEM is operated under vacuum, therefore all samples need to be dried. The biological samples cannot be imaged directly and need complexed processing steps before being imaged. The dehydration of biological samples on the surface of graphene will affect the structure of the biological samples.

Scanning electron microscopy (SEM) imaging were performed under secondary electron mode using a Zeiss Leo (1525) system with an accelerating voltage of 5 kV. A normal operating vacuum of 2×10^{-5} mbar was achieved during the pump down of the chamber. The graphene on the substrate was mounted on the metallic sample holder using carbon tape. The samples are coated with 15nm chromium using a turbomolecular pumped sputter coater (EMS150T Plus) before performing the SEM microscopy.

Images obtained were analyzed using ImageJ [226] to obtain particle size and density on the graphene surface. Images were processed using a colour threshold with the threshold colour set as B&W and colour space as RGB, with the same threshold value applied. Then, the particles were counted using the “analyze particles” toolbox in ImageJ.

3.2.5 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) is a powerful technique for analysing the surface chemistry of a material. It provides both quantitative information about the elemental composition and chemical bonding of the top few nanometres of the surface of a material. The spatial resolution of XPS is between three to a few hundred μm . XPS is particularly useful in characterizing the degree of surface functionalization of graphene. It can identify the element present on the surface of graphene before and after surface functionalization, providing information of the presence of specific functional groups. The basic XPS instrument is composed of an x-ray source, and sample stage, extraction lenses, an analyser and a detector. (Figure 3-5) The operation of XPS is performed under ultra-high vacuum condition.

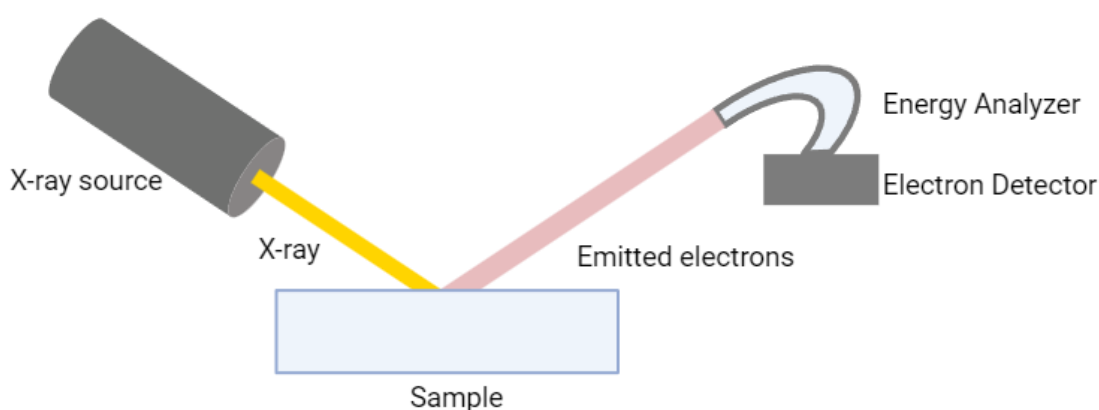


Figure 3-5 Schematic of a basic XPS instrument set-up. The x-ray source generated x-rays will irradiate onto the sample and produce photoelectrons. These photoelectrons will be detected and analysed.

The working principal of XPS is based on the photoemission spectroscopy of a sample when the sample is irradiated with a beam of X-rays. (Figure 3-6) The X-ray photons from the source interact with the core level electron of an atom, leading to a complete transfer of photon energy to the electron. The electron, which is referred as the photoelectron, will then have sufficient energy to escape from the surface of the sample. Through determination of the kinetic energy (KE) of this photoelectron using the spectrometer, the binding energy (BE) of the core electron can be determined. The binding energy is determined using the following equation:

$$BE = h\nu - KE - \phi_{spec}$$

Where BE is the binding energy of the electron, KE is the kinetic energy of the emitted electron, $h\nu$ is the X-ray photon energy (typically 1486.6 eV for Al K α and 1253.6 eV for Mg K α , which are the two most commonly used X-ray sources), and ϕ_{spec} is the work function of the spectrometer. Each element has its characteristic electron binding energy, through analysing the electron binding energy, the element can be identified. It is worth noting that XPS is able to detect all elements besides hydrogen. Through analysing the binding energy and the intensity of the peak of photoelectron, the chemical state and quantity of the detected element can also be determined.

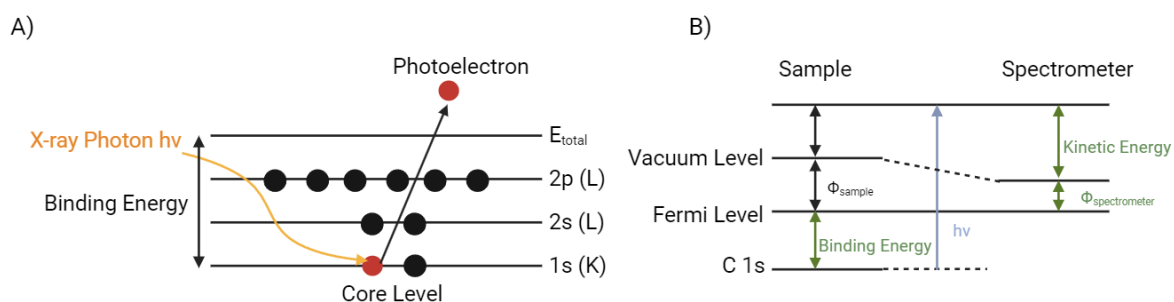


Figure 3-6 A) Schematic diagram of processes of an emission of a photoelectron resulting from x-ray incident on surface. B) Energy level diagram of the schematic diagram of the basic XPS equation.

XPS is widely in characterizing the surface chemistry of carbon-based materials. The C1s spectra of a hexagonal lattice with sp² carbon atoms show a characteristic peak at 284.6 eV on the C1s spectrum. When lattice defects are introduced, sp³ carbon atoms will be introduced, with peaks at 285 eV. The XPS analysis of the carbon spectrum allows the analysis of multiple bonds, e.g., sp² carbon, carbonyl, carboxyl, epoxy, etc. The XPS analysis can confirm the presence of all the chemical functional groups involved in the surface functionalization steps. Meanwhile, the analysis of N 1s spectrum from the XPS can confirm the presence of the covalently immobilized antibodies and the relative surface coverage of antibodies on the surface of graphene.

Although XPS is a simple and straightforward surface analysis technique for the acquisition of nondestructive quantitative information of surface chemistry. However, the accuracy of the elemental composition and chemical state of the elements present on the surface of a material is up to 0.1 at%. When the surface elemental analysis with higher precision is required, XPS need to be used in conjunction with other techniques.

XPS experiments were performed with a Thermo Fisher K-Alpha+ X-ray Photoelectron Spectrometer (XPS) system using a monochromatic Al radiation source ($h\nu = 1486.6$ eV). All measurements were performed in an ultra-high vacuum chamber for spectroscopic analysis with a base pressure of 5×10^{-8} mbar.

3.2.6 Raman Spectroscopy

Raman microscopy is a powerful technique for characterizing molecular vibrations, offering detailed insights into molecular composition, structure, and interactions. It is widely used to investigate the physical properties of graphene. Raman spectroscopy can provide both qualitative and quantitative information of graphene, including properties related to the thickness of graphene layers, the quality and the presence of defects in graphene, etc.. Raman

spectroscopy relies on the detection of Raman scattering from the sample, which is an inelastic scattering process.

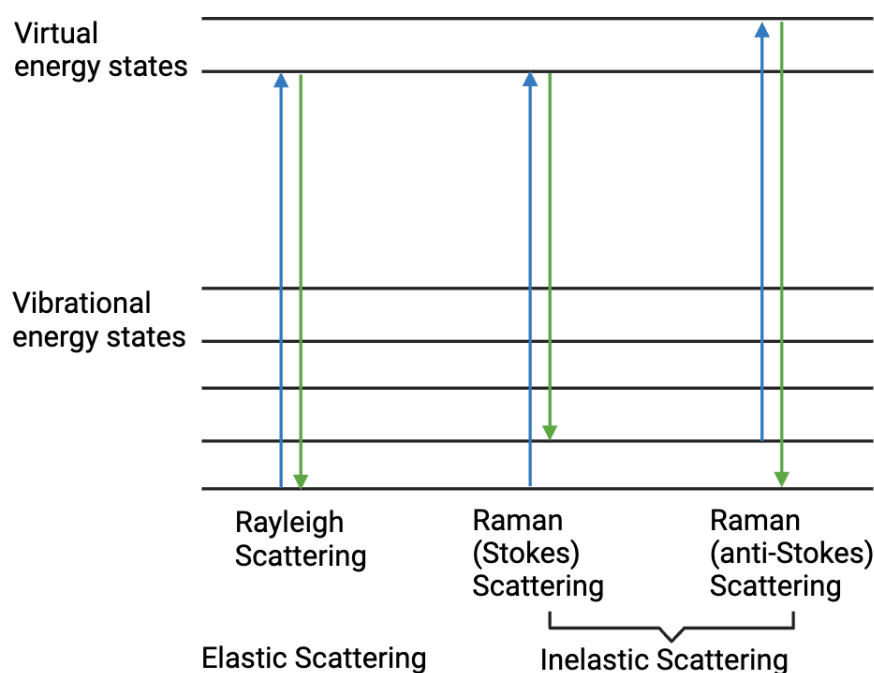


Figure 3-7 Energy-level diagram of the elastic and inelastic scattering mechanisms and the states involved in Raman spectra.

Typically, a monochromatic light source is used, the excitation light source used in this thesis is laser. The incident photons interact with the sample and induce the polarisation of the molecular electron in the sample, resulting in the energy of the photon transferred to the molecule, the energy of the photons can be shifted up or down. If the total energy of the system remains unchanged, the energy of the scattered photon is equal to the energy of the incident photon. This scattering is elastic, Rayleigh scattering will occur. If the scattering is inelastic, Raman scattering (Stokes or anti-Stokes) is dominant. In the case of a Stokes scattering, the molecule gains energy from the incident photon and excited to a higher vibrational state, the scattered photon loses energy and has increased wavelength. On the other hand, in the case of an anti-Stoke scattering, the molecule loses energy and shifted down to a lower vibrational state, the scattered photon gains energy and has decreased wavelength. (Figure 3-7)

Raman spectroscopy filtered out the elastic scattered Rayleigh scattering using a notch filter, edge pass filter, or a band pass filter. It collected and dispersed the rest of the scattered light by the detector. A Raman spectrum demonstrates the plot of intensity as a function of the Raman frequency shift, which are typically reported in wavenumbers in cm^{-1} ($1/\lambda$).

Raman spectroscopy is very sensitive that it can differentiate small differences between geometric structures and bonding within molecules. The Raman spectrum shows the vibrational spectroscopic fingerprint of the sample, which allows for the determination of the structural defects and chemical doping effects. The typical Raman spectra of a monolayer graphene is shown in Figure 3-8. The spectra are consisted of two principle bands, known as the G and 2D-bands. If defects are present in the carbon lattice in graphene, a D-band may also present in the Raman spectra. The D-band of graphene appears at approximately 1350 cm^{-1} . The G-band appears at approximately 1583 cm^{-1} . The 2D bands is the second order or the overtone of the D-bands. This band is used for the determination of the thickness of graphene layer. When the sample is a single layer graphene, the 2D-band is expected to be a single symmetric peak, with a full width half maximum of around 30 cm^{-1} . Multiple layer graphene will lead to band splitting, causing the 2D band appear with lower symmetry. Meanwhile, the peak intensity ratio of the 2D and G-bands is another reference for the determination of the overall quality of graphene layer. The I_{2D}/I_G peak intensity ratio for a high quality, monolayer graphene is expected to be around 2. The lack of presence of D-band, along with a sharp symmetric 2D band and its intensity relative to the G band act as a good indicator for the quality and defect density of the graphene sample. Therefore, Raman spectroscopy is an powerful and useful tool for the thickness of graphene layer and graphene quality characterization.

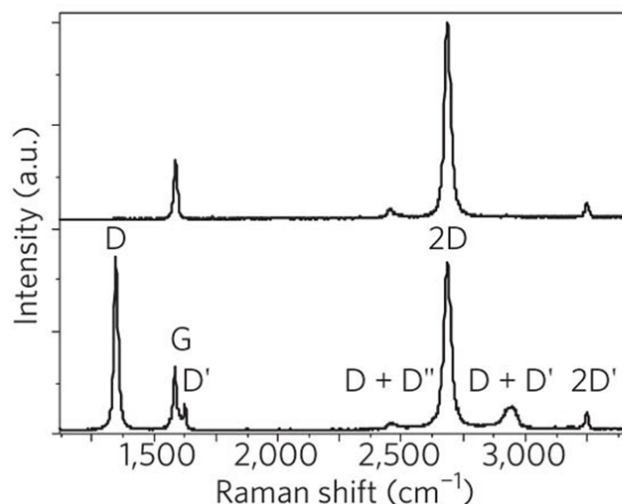


Figure 3-8 Raman spectrum of pristine (top) and defected (bottom) graphene, with main peaks labelled. Single layer graphene can be identified by the intensity ratio of the 2D-band to G-band.[229]

For the characterisation of GFET samples in this thesis, Raman spectroscopy measurements were performed on both single Raman spectra and Raman maps. The obtained Raman spectra were used to characterise the chemical and structural properties of graphene samples before and after functionalization. All Raman spectroscopy were performed in air under ambient conditions at room temperature. Raman spectroscopy measurements were performed using either a Thermo Fisher Scientific Inc. DXR™2xi or a LabRAM HR Evolution Raman spectrometer (Horiba Scientific) and excited with laser (Torus MPC 3000) with a wavelength of 532nm (excitation energy $E_L = h\omega_L = 2.33 \text{ eV}$) through an optical fibre, and an objective lens of 100x, NA = 0.8, and a laser spot of $0.4 \mu\text{m}$. The laser power was kept below 2 mW and the diffraction grating was 600mm/grove. The collected Raman spectra range spanned the wavenumber region of $1200 - 3000 \text{ cm}^{-1}$. The Raman peak position was calibrated based on the first order Raman signal of Silicon, at 520.7 cm^{-1} . The D, G, and 2D peaks were fitted with Lorentzian functions.

3.2.7 Electrochemical Impedance Spectroscopy (EIS)

The electrochemical impedance spectroscopy measurements (EIS) or cyclic voltammetry (CV) measurements were performed with SP-300 Biologic Potentiostat, with an ac input of 10 mV and varying additional dc voltages (from -0.3 V to 0.3 V) as the gate bias. A sweep rate from 50mV/s to 500mV/s was employed. Gold (Au) and Ag/AgCl were used as the working electrode and the reference electrode respectively, a 1cm × 1cm graphite sheet was selected as the counter electrode. During the EIS measurement, all three electrodes were immersed in the PBS solution for the measurement. Different dilutions of PBS (d1000, d100, d10 and 1xPBS) were selected as the electrolyte solution.

3.2.8 Contact Angle Measurements

Contact angle measurements were conducted with an optical tensiometer (Biolin Scientical) under ambient condition (22°C). Sessile drop technique was selected for the characterization of wetting. A small droplet (6-15 µl) of water was deposited on the substrate and the contact angle was measured within 5 seconds. A baseline was constructed in the software to define the base of substrate, with a Laplace-Young's curve fitted for obtaining the contact angle.

3.2.9 Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS)

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a very precise technique for the surface analysis. It can provide the surface chemical composition and the molecular information on the surface of graphene with a sampling depth of less than 2 nm, a spatial resolution of around 0.1 µm and a detection limit in the ppm range (on the order of 0.1% - 0.01% atomic concentration). Therefore, offers a more precise and sensitive platform for the analysis of small features and compositional variability within the biomolecules on the surface of graphene.

During the TOF-SIMS analysis, a pulsed and focused primary ion beam (usually Cs or micro focused Ga) removes molecules from the outermost surface of the sample. The particles removed from the top several monolayers of the sample surface, referred as secondary ions.

These secondary particles are then accelerated into a “flight tube” towards a detector. The masses of these particles are determined by measurement of the exact time of these particles reach the detector (i.e. time-of-flight). The basic components of a TOF-SIMS instrument are shown in Figure 3-9. There are three operational modes available with TOF-SIMS analysis: surface spectroscopy, depth profiling and surface imaging.

It is worth to note that TOF-SIMS is not a technique for the absolute quantitative analyses of graphene surfaces. It is a useful tool for the semi-quantitative analysis and comparison of graphene biosensors with different surface modifications. Meanwhile, the dataset obtained from TOF-SIMS is very complicated. The data analysis and interpretation for TOF-SIMS results is time-consuming and critical. Multivariate analysis for the interpretation of TOF-SIMS datasets have been widely applied. These Multivariate statistical analysis technique includes principal components analysis (PCA), neural networks (NN), multivariate curve resolution (MCR), mixture models (MM), and maximum auto-correlation factors (MAF). Among all of these Multivariate analysis techniques, PCA is the most widely applied method and is therefore used for the analysis of TOF-SIMS data in this thesis. Details of PCA and PCA analysis of TOF-SIMS data will be introduced in section 4.4.4.

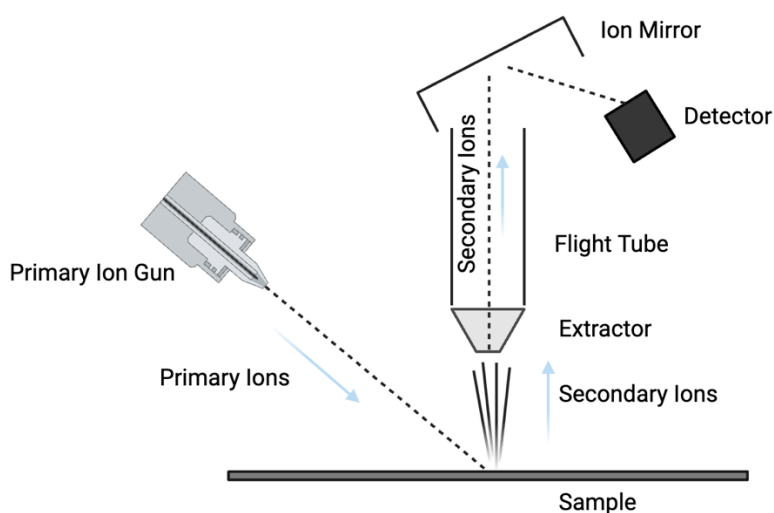


Figure 3-9 A schematic diagram of the basic components and the fundamental principles of ToF-SIMS analysis. The emitted primary ions from the primary ion gun will bombards the specimen and generate secondary ions from the top several monolayers of the sample surface. These generated secondary ions will be detected and analysed.

TOF-SIMS analysis was conducted using the TOF.SIMS 5 (ION-TOF GmbH) instrument equipped with Bi_3^+ ion clusters (25 keV liquid metal ion gun). Positive ion high mass resolution TOF-SIMS spectra were acquired from at least ten non-overlapping $100\ \mu\text{m} \times 100\ \mu\text{m}$ areas for each sample (at an applied resolution of 128×128 points). The primary ion dose density deposited on the surface was kept identical for all measurements and below $10^{12}\ \text{ion}/\text{cm}^2$ for a static TOF-SIMS condition. A current of about 0.5 pA was applied for all measurements. Mass calibration was performed with H^+ , H_2^+ , CH^+ , C_2H_2^+ and C_4H_5^+ peaks, before performing further analysis. A minimal mass resolution ($m/\Delta m$) > 8000 at C_4H_5^+ was obtained. For the investigation of antibody orientation on the surface of graphene, a list of 40 characteristic positive ion fragments for amino acids were selected and examined. Details on the principal of analysis of surface antibody orientation using TOF-SIMS with PCA are described in section 4.

3.3 Fabrication of graphene field effect transistor sensor array

Standard microfabrication process steps were used to fabricate GFETs sensor array. This includes sample cleaning, patterning, etching, metallisation, and passivation. The process steps are as follows.

3.3.1 Cleaning and graphene modification.

After the completion process of graphene transfer onto 90 nm SiO_2 substrates, a general cleaning using acetone, IPA, and DI water for 10 minutes each step was performed to remove organic residues on the substrate. The samples were immersed in HMDS for 3 to 6 hours to improve the adhesion to the substrate before photoresist and reduce the effect of residues from photoresist on graphene. [143]

3.3.2 Graphene patterning and etching.

AZ5214E photoresist was spun on the substrates at 500 rpm for 45 seconds to form film with thickness of 1.3 μm on graphene. Then, the samples were baked at 90 °C for 5 minutes and placed under a mask and exposed to UV light of around 85 mJ/cm² before they developed for 30 seconds with MF319 developer. After that, the samples were placed in Reactive Ion Etching chamber in the presence of oxygen for 30 seconds where exposed graphene was etched. Photoresist was removed using acetone, and IPA followed by DI water rinsing.

3.3.3 Contact opening and metallisation.

The samples were cleaned as in step 3.3.1. After that, another photolithography step was applied. In this step, photoresist was used as image reversal. After photoresist development, the samples were placed in HMDS for over 12 hours to improve the contact resistance. [143] Sputter system was used to deposit a 5 nm/60 nm of Ti/Au. It was followed by lift-off step to remove the photoresist.

3.3.4 Device passivation.

To minimise parasitic effects and passivate the devices during sensing measurements, an SU8 film of 1.7 μm was spun coat and patterned on the substrates leaving only the sensing area of the graphene exposed. This was followed by a hard baking step at 130 °C to harden the SU8 film.

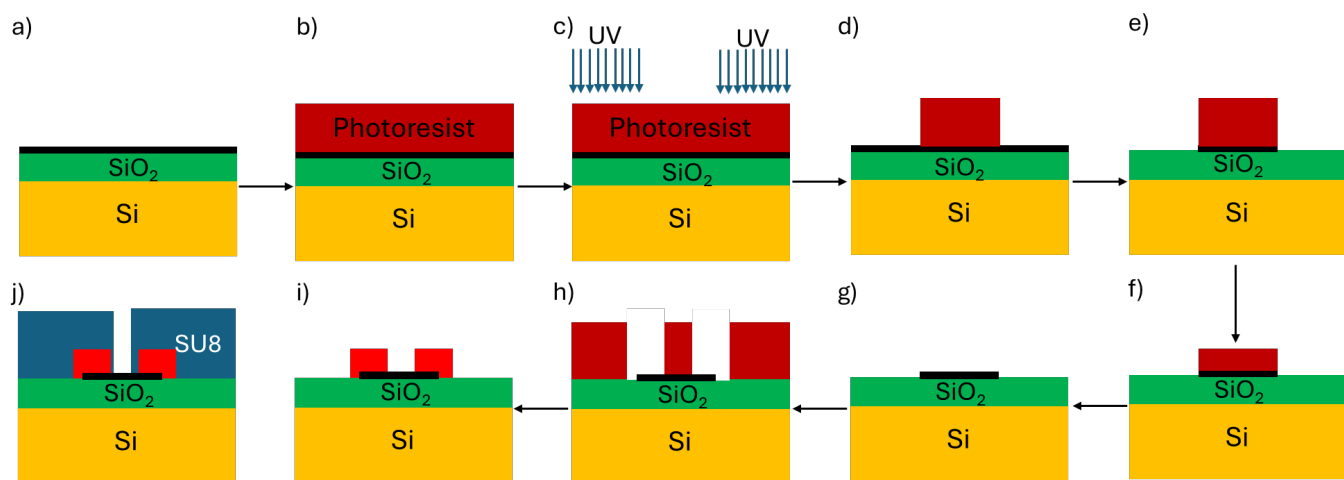


Figure 3-10 Schematic representation of the GFET fabrication steps. The main GFET fabrication steps involves: sample cleaning, patterning, etching, metallisation, and passivation. After the sample cleaning, b) photoresist was spun on the substrates; c) the samples were baked at 90 C for 5 minutes and placed under a mask and exposed to UV light; d) the samples were placed in Reactive Ion Etching chamber to etch the undesired graphene; e-f) Photoresist was removed using acetone, and IPA followed by DI water rinsing; h) photolithography step was applied for image reversal; i) Sputter system was used to deposit a 5 nm/60 nm of Ti/Au, followed by lift-off step to remove the photoresist; j) an SU8 film of 1.7 μm was spun coat and patterned on the substrates leaving only the sensing area of the graphene exposed.

3.4 Preparation of biological fluids

3.4.1 Phosphate Buffered Saline (PBS) Solutions

PBS buffer solution is a widely used isotonic buffer solution. It is particularly used in biosensor applications to maintain the pH and osmolarity of the solution, providing a stable and biologically compatible environment for sensing applications.

PBS solution was prepared using pouch (Sigma-Aldrich). When one pouch is dissolved in one liter of DI water, a 1 $\times$ PBS solution (0.138 M NaCl; 0.0027 M KCl) is yielded. This solution is also known as 10 mM PBS with pH 7.4.

PBS solutions were made up with a range of concentrations: $\times 1000$ diluted PBS - 1 $\times$ PBS. Concentrations of PBS solutions were prepared by 10-time serial dilution of 1 $\times$ PBS solution with DI water.

3.4.2 Bovine Serum Albumin (BSA)

BSA is a versatile globular protein present in the blood serum of cow. BSA has a chemical similarity to human serum albumin. It is widely used in biosensor applications to block the non-specific binding sites on the surface of sensors.

BSA solution was prepared using lyophilized BSA powder (Sigma-Aldrich). 1% BSA solution was prepared by dissolving BSA in 1 × PBS solution at pH 7.4 and stored at 4°C for later use.

For the electrical measurements, samples were blocked using 1% bovine serum albumin (BSA) in 1 × PBS at room temperature for 1 h, then rinsed with PBS and DI water, and dried with N₂. The prepared samples were stored at 4°C for later use.

3.4.3 Exosomes

Lyophilized exosomes from plasma of healthy donors or cancer cell line were purchased from HansaBioMed Life Sciences Ltd or Abacam and stored at 4 °C.

The working concentrations of tested exosomes were prepared by a 10-time serial dilution from the stock solution. Based on the selected electrolyte buffer solution, × 1000 diluted PBS was used as the solvent. Further details on the selection of exosomes and its preparation are discussed in each section.

3.4.4 Clinical samples

Patient samples were obtained through the *CIRcular and Non-coding RNAs as Clinically USEful Biomarkers in Pancreaticobiliary Cancers* (CIRCUS) clinical trial at Royal Surrey County Hospital NHS Foundation Trust (NCT04584996). Research Ethics Committee (REC) approved, IRAS Project ID: 277406.

All patients planned for surgical resection for PDAC were identified through the HPB multi-disciplinary team (MDT) meeting and by the clinical team at Royal Surrey County Hospital NHS Foundation Trust. These patients were approached either in clinic or at the pre-operative assessment. Plasma samples were taken from resectable PDAC patients and those with locally

advanced, borderline, or metastatic PDAC deemed unsuitable for surgical resection and for potential medical treatment (*i.e.*, neo-adjuvant or palliative chemotherapy). The control group consisted of patients diagnosed and/or due to undergo surgery for benign pathology (*e.g.*, gallstones, chronic pancreatitis, *etc.*)

Exclusion criteria for the patients are: unwilling or unable to provide written informed consent, non-English speaking, known to be pregnant, aged <16 years, known diagnosis of HIV or Hepatitis B/C virus.

Blood sample (total 30mL) were drawn into anticoagulant treated EDTA collection tubes, labelled, and placed on ice. Blood samples were centrifuged within 2 hours of collection using a validated double spin operating method for plasma isolation by Oxford University Trust. Samples undergo 2 steps of spin. The samples were centrifuged at 800× g at 21°C for 15 min, followed by the second centrifugation at 4000× g at 21 °C for 15 min. Following the centrifugation, samples were immediately transferred into clean aliquots, and frozen at -80°C.

3.5 Functionalization of GFET biosensor

Biofunctionalization of the GFET biosensor mainly includes two steps of incubation, firstly with the linker molecule that can bind to graphene, followed by a step of antibody conjugation through the formation of covalent amide bonds with the linker molecules. Two different linker molecules, tetrakis(4-carboxyphenyl) porphyrin (TCPP) or 1-pyrenebutanoic acid succinimidyl ester (PBASE), were employed for the non-covalent functionalisation of graphene. The selected linker molecules can bind with the graphene via π - π interaction. Solvent, such as 2-methoxyethanol and dimethylformamide (DMF) are used to prevent the hydrolysis. The following section details these two surface modification techniques.

3.5.1 TCPP Method

Firstly, the graphene substrates were incubated with TCPP (50 μ M) (Tokyo Chemical Industry) in 2-methoxyethanol (Tokyo Chemical Industry) for 2 hours at room temperature for spontaneous non-covalent functionalisation. Then, samples were gently rinsed with 2-

methoxyethanol and 1 × PBS to remove excessive TCPP from the graphene surface, and then dried with N₂. The carboxyl groups on TCPP were activated via EDC/NHS chemistry for 30 min at room temperature with a mixture of 200 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC/HCl) (Sigma-Aldrich) and 50 mM of N-hydroxysuccinimide (NHS) (Sigma-Aldrich) in DI water. Then, samples were rinsed with 1×PBS and DI water and dried with N₂.

3.5.2 PBASE Method

PBASE solutions were made up by dissolving PBASE (Sigma-Aldrich) in dimethylformamide (DMF) (VWR International or Sigma-Aldrich).

The graphene substrates were then incubated with the PBASE solution (3 mM) for 2 hours at room temperature for spontaneous non-covalent functionalisation. Then samples were gently rinsed with DMF to remove excess PBASE from the surface of graphene, before being rinsed with DI water and dried with N₂.

3.5.3 Antibody Conjugation

Different antibodies were selected according to various purposes of its application. The selected antibodies were immobilized by covalent bonding on the TCPP- or PBASE-functionalized graphene surfaces.

The antibodies used were supplied in an aqueous buffer solution and were diluted using 1 × PBS to the desired concentration. Droplets of 20 µL of antibody or antibody fragments were placed on the graphene surface and left overnight in a humidified environment at 4 °C for incubation. The samples were then sequentially rinsed with 1 × PBS and DI water and dried with N₂. The prepared samples were stored at 4°C for later use.

The details about antibodies selected and their immobilisation process are introduced below. For the investigation of the effect of linker molecule on antibody orientation and performance of GFET biosensors:

To investigate the surface binding efficiency of antigens to surface-immobilized antibodies, the anti-CD63 antibody (BD Biosciences, #556019) or anti-GPC-1 antibody (Invitrogen, PA5-28055) were selected for an exosome binding investigation.

To study the surface orientation of immobilized antibodies, polyclonal goat anti-rabbit IgG (Thermo Fisher, # 31210) was immobilized on TCPP- and PBASE-functionalized graphene surfaces. F(ab')₂ (Thermo Fisher, # 31234) and Fc fragments (Thermo Fisher, # 31216) of the IgG were immobilized on functionalized substrates as reference substrates. The antibodies used were supplied in an aqueous buffer solution and were diluted using 1 × PBS to a concentration of 500 µg/mL.

For the investigation of the performance of GFET biosensors for clinical detection of PDAC cancer exosome in patients:

The anti-CD63 antibody (BD Bioscience US) or anti-GPC-1 antibody (Invitrogen PA5-28055) were used. The antibodies were supplied in a stock solution of 0.5 mg/mL in an aqueous buffer solution (containing ≤ 0.09% sodium azide) and were diluted using 1 × PBS to a concentration of 100 µg/mL.

3.6 Device Testing

3.6.1 Portable readout electronic system

Electrical characterization of the different graphene sensing units contained inside the GFET chip array was performed using a custom-designed and portable readout system with a dedicated graphical user interface (GUI) connected to Matlab (Mathworks Inc., USA) *via* a Universal Serial Bus (USB).

The portable readout system employed time-multiplexed signal acquisitions (MUX) from the 12 channels connected to each graphene sensing unit, whereby the individual drain-source currents (iDS) were detected in a voltage-divider circuit topology, amplified, and digitized by a high-speed and high-resolution analogue-to-digital converter (ADC) connected to the central microcontroller (MCU) on the embedded electronics. The drain-source voltage level (vDS) on the graphene channels is imposed by the user during the iDS measurements through

the GUI, which converts the digital value into an analogue equivalent level using digital-to-analogue conversion (DAC) circuitry within the range $\pm 8\text{V}$ and resolution of $250\text{ }\mu\text{V}$. For measurement of the $i\text{DS} - v\text{GS}$ curve, also known as Dirac's point, an additional DAC is employed to provide an automatic voltage level sweep across the floating gate and within the range specified by the user. $i\text{DS}$ measurements in both stimulation modes (including extended temporal measurements) can be applied to a single graphene channel or to all twelve channels at once, with the true value for $i\text{DS}$ being calculated by a Fast Fourier Transform (FFT) algorithm applied to the samples acquired by the ADC, thereby achieving a current resolution of 20 nA and bandwidth of 15 kHz .

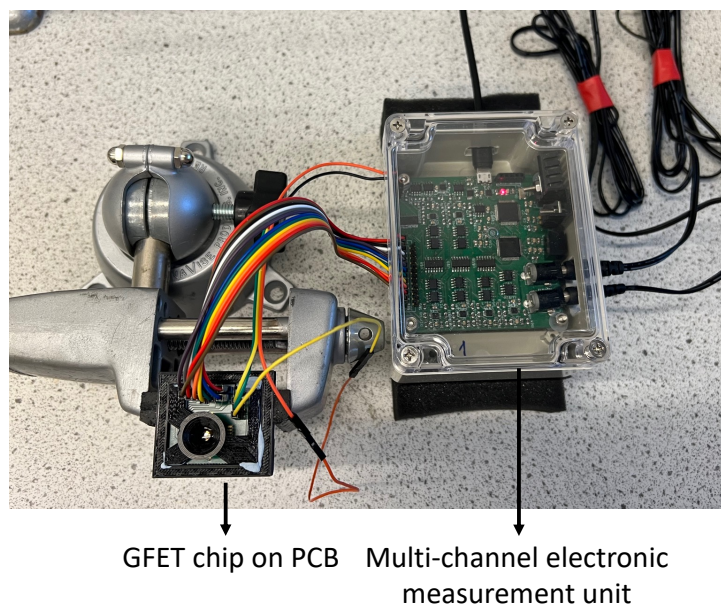


Figure 3-11 Illustration of the testing device with electronic readout measurement unit. The electronic readout measurement unit is placed inside the transparent box shown on the right hand side of the image.

3.6.2 Electrical measurements

Measurement performed with Keithley source meter:

Immediately following the cleaning and functionalization of developed GFET device, electrical measurements were performed using a Keithley source meter (Keithley 2636B analyser) with

transfer curves recorded. The source-drain voltage was fixed at 20 mV, and the electrolyte gate was swept from -0.4 to 0.4V at a sweeping rate of 5 mV/s, with a delay of 0.05 s. Phosphate-buffered saline (PBS) buffer solutions with different serial of dilution, 0.001 x PBS (d1000 PBS for low ionic strength) and 0.1 x PBS (d10 PBS) solution, is used for the electrical measurements.

Measurement performed with the portable electronic readout system:

Immediately following the functionalization and antibody conjugation of the GFET biosensor, electrical measurements were performed in 0.001 × PBS (×1000 diluted PBS to ensure a solution with low ionic strength) using a portable electronic readout system.[230] The source-drain voltage was fixed at 0.1 V, and the electrolyte gate was swept from 0.4 to 0.9 V at a sweeping rate of 2 mV/s, rendering source–drain currents in the order of tens of microamperes (μA) in ×1000 diluted PBS. Samples were tested using the developed on-chip GFET sensors with all characteristic I-V transfer curves recorded. For the laboratory investigation of the analytical sensing performance of the GFET biosensors in the PBS buffer solution, MCF-7 exosomes were used as model exosomes, the working concentration of exosomes (MCF-7 exosomes, Abcam, ab239691) was prepared by 10-time serial dilution from the stock solution, and ×1000 diluted PBS was used as the solvent. For clinical validation with PDAC patients and healthy control plasma, ×1000 diluted PBS was selected as the buffer solution. The transfer curves in the buffer solution were recorded first. Then, the plasma sample (20 μL) was added to the functionalized sensor surface for 15 min of incubation. The GFET chips were then rinsed multiple times with PBS (3× times of 200 μL) and DI water. Finally, the sensor immobilized with exosomes in plasma was tested in the buffer solution. Total testing time is 45 mins that include the 15 mins incubation, multiple rinsing and 24 mins for two measurements.

3.6.3 Electrical Data Analysis

The sensing and working principal of the GFET biosensors relies on the measurement of the electrical current in the graphene device channel. The electrical current of the GFET biosensors is controlled through the potential applied between the source, drain and the gate

electrodes. There are three representative curves that characterize the GFET sensors: transfer curves (current I_{ds} – gate bias V_g), time series (current I_{ds} – time t) and output curves (current I_{ds} – drain-source bias V_{ds}). Transfer curves (I_{ds} – V_g) and output curves (I_{ds} – V_{ds}) were used as the characteristic electrical measurements of the GFET sensors in this thesis.

During the measurement with transfer curves, a series of shifted I-V curves are obtained with different buffer solutions or different concentrations of exosomes. The transfer curves are measured with the gate voltage V_g sweeping between a set range, while a fixed bias V_{ds} between the source and drain electrodes is controlled. The I_{ds} – V_g curves measured demonstrated a “V” shaped curve, as shown in Figure 3-12. There are several key features that can be extracted from the transfer curves, including the Dirac point or charge neutrality point (CNP) V_{CNP} , the transconductance g_m , the maximum current I_{max} , and the minimum currents I_{min} . These extracted key features can be used as the indicator for sensing. The key parameters will be discussed in detail below.

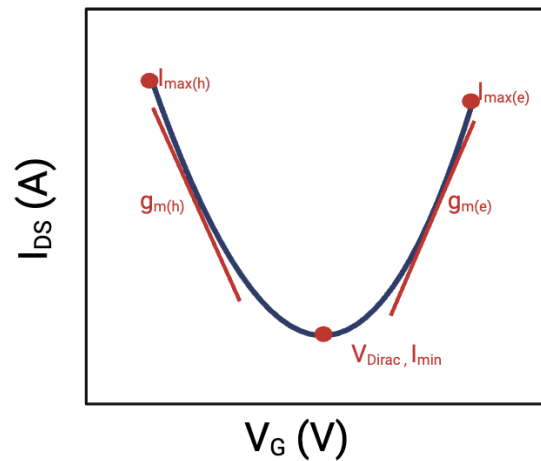


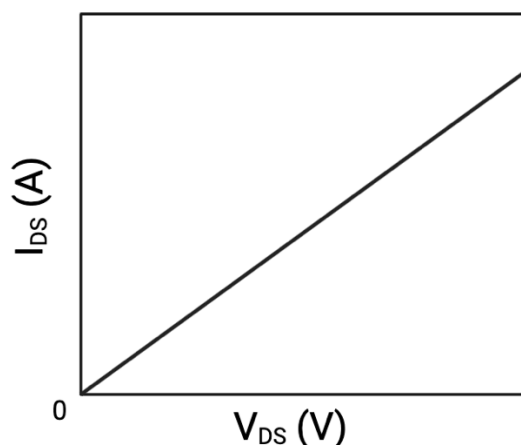
Figure 3-12 Characteristic transfer curves, I_{ds} – V_g curve, of a GFET, with key features illustrated, including the Dirac point or charge neutrality point (CNP) V_{CNP} , the transconductance g_m , the maximum current I_{max} , and the minimum currents I_{min} .

The dip of the “V” represents the minimum conductivity called the Dirac point or charge neutrality point (CNP) (V_{CNP}). The shift of the Dirac point ΔV is the most commonly used feature for the GFET biosensors. The Dirac point ΔV of the GFET biosensor is related to the level of doping of the graphene. During the biosensing measurements, the shift of the Dirac

point occurs due to the interaction between the target bioanalyte present in the electrolyte solution and the immobilized biorecognition molecules on the surface of the graphene. This interaction changes the doping level of graphene and the Fermi level of graphene, therefore leading to a change in the Dirac point ΔV . The value of the shift in the Dirac point ΔV can also be used in correlation with the target bioanalyte concentration to monitor or predict the concentration of the target bioanalyte presented.

The transconductance g_m , is another important feature in graphene biosensing. It is defined as the maximum slope of the linear part of the p- or n-branch of the transfer curves, $g_m = \Delta I_{ds} / \Delta V_g$. The transconductance of the GFET biosensor can reflect the performance of the GFET biosensor, it mainly depends on the mobility of graphene. The mobility of charge carriers in graphene is related to the amount of impurities present in the GFET. During the biosensing measurements, the target analyte may introduce additional scattering sites to the graphene, increasing the amount of impurities, and leading to a change in the transconductance of the GFET biosensor.

The output curves of the GFET are obtained with buffer solutions prior to the measurements with exosomes. The output curves are obtained as a function of the drain–source voltage V_{ds} with the gate voltage V_g fixed. The shape of the output curve can be used as an indicator for the quality of graphene, when the quality of the fabricated GFET biosensor is good, a linear ohmic curve is expected, as shown in Figure 3-13.



3.6.4 Microfluidic Channel Fabrication and Integration

Traditional methods for the fabrication of microfluidic channels are time-consuming and costly, involving the use of photolithography and soft lithography. Three-dimensional (3D) printing has been explored as an alternative technique for microfluidic channel fabrication. 3D printing, also known as additive manufacturing, is an emerging technique that creates a physical object by layering and fusing thin layers of materials on top of itself, following a digital design. 3D printing offers more accessible and flexible options for direct or indirect fabrication of multilevel and multilayer microfluidic devices. The use of 3D printing allows the rapid prototyping and customization of devices.

In order to perform testing with liquid using a liquid gate GFET, a custom 3D printed microfluidic channel was implemented. This microfluidic channel can be mounted on top of the fabricated GFET devices and act as a reservoir for the containment of liquids on the surface of GFET sensor. To avoid degradation and reduce leakage currents, it is important to ensure the metal contact on the GFET sensor is isolated from liquids. Meanwhile, the position of the liquid needs to be consistent for repeated testing to enhance signal-to-noise ratio and reduce parasitics. Therefore, the channel was designed to be fixed at the center of the chip such that the drain and source electrodes are protected from liquid. An external 3D printed base with clamps were used for insurance of the fixed position of liquid.

All components were designed using the computer aided design (CAD) program SOLIDWORKS. The 3D printing of components was achieved using an Ultimaker 2+ 3D printer (Ultimaker, USA). The printer was specified with the capability of resolving extruded features as 50 μm and intruded features as small as 100 μm .

Figure 3-14 illustrates the process flow for fabrication of microfluidic devices using 3D printed parts. Both direct and indirect 3D printing techniques were used. 3D printed transfer moulding (PTM) is used to create the internal Polydimethylsiloxane (PDMS) ring for the microfluidic channel. The elastic and soft PDMS ring can adhere to the GFET surface without

damaging the graphene and metal contacts. This PDMS ring acts as a connection piece and sealant between the top reservoir and the GFET surface. A component mould is designed and used as a negative mould to cast the PDMS ring. PDMS is applied, cured and released from the mould. The base, top reservoir, clamps and component moulds are fabricated via a direct 3D printing process. (Details are illustrated in

Figure 3-14 B)

A negative mould was designed with two parts. One base part with the inverse feature of the desired shape. Another top part to cover the base and mould the feature, the top part is also used to facilitate the removal of cured PDMS. PDMS was prepared using the PDMS kit (Sylgard 184 Elastomer Kit, Dow Corning, Midland, MI, USA). The kit is supplied in two parts, one base which is a vinyl containing resin and one curing agent which is a hydrosiloxane copolymer curing agent. These two parts are mixed with the standard ratio of 10:1 base to curing agent. The PDMS mixture was then degassed using a vacuum chamber to 10^{-4} torr or a centrifuged at 40,000 rpm for 15 minutes. The mixture was then carefully poured on the 3D printed mould sprayed with the mould release (Ambersil, UK). The filled mould was then left to cure at room temperature for 24 hours or in a 60 °C oven for 4 hours. The cured PDMS ring was carefully removed from the mould by manually peeling the PDMS from the mould and the excess PDMS was trimmed. The fabricated PDMS ring was rinsed using acetone, isopropanol and DI water. Finally, the base, top microfluidic reservoir, PDMS ring and clamps are assembled. This acts as the microfluidic device for the GFET devices. The design of the microfluidic channel ensures the liquid position and contact of the liquid with graphene only. The use of 3D printing enables fast, cheap and reliable fabrication of microfluidic devices. The fabricated microfluidic devices can be used for multiple times for multiple devices.

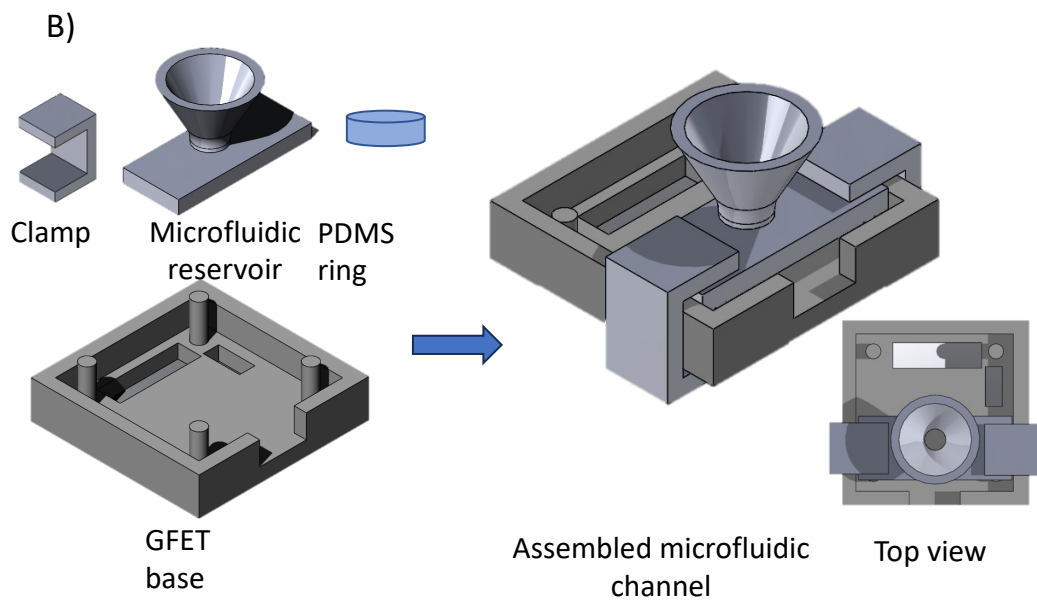
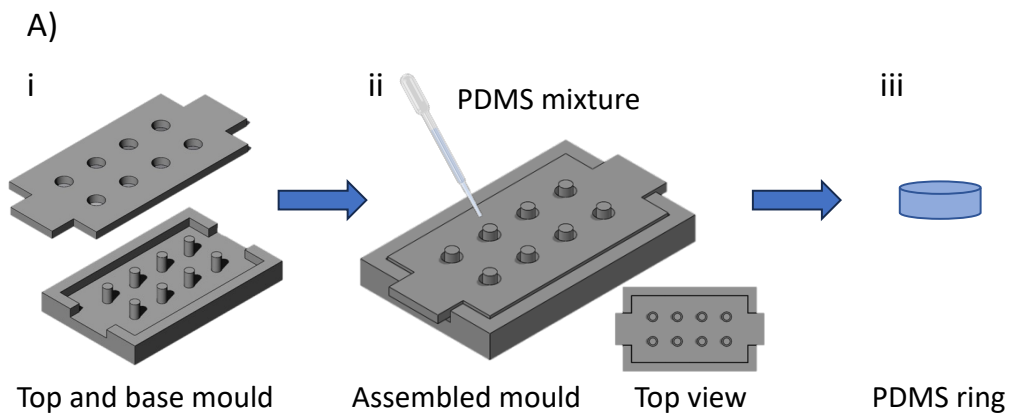


Figure 3-14 Schematic diagram of the step-by-step fabrication of steps for the microfluidic channel. A) Schematic process for the fabrication of PDMS ring. i) Negative mould with 2 parts are 3D printed with the desired PDMS ring size. ii) The negative mould was assembled. The PDMS mixture were mixed at the desired ratio and ultracentrifuged to remove bubbles. The prepared PDMS mixture was carefully poured into the mould and left for curing overnight. iii) The PDMS ring was then removed from the mould, trimmed, and cleaned for further use. B) Schematic diagram of the CAD designed 3D printed parts of the microfluidic channel. These printed parts and the PDMS ring were inserted under the microfluidic reservoir for the assembly of the microfluidic channel for GFET testing.

3.6.5 Clinical testing protocol

Immediately following the functionalization and immobilization of the GFET biosensor, electrical measurements were performed in 0.001 × PBS (×1000 diluted PBS to ensure a solution with low ionic strength) using a portable electronic readout system.[230] GPC-1 antibody was functionalized on the surface of the GFET biosensor for all the electrical measurements. Source-drain voltage was fixed at 0.1 V, and the electrolyte gate was swept from 0.4 to 0.9 V at a sweeping rate of 2 mV/s (the reading time per channel is around 1 min), rendering source–drain currents in the order of tens of microamperes (μA) in ×1000 diluted PBS. Samples were tested using the developed on-chip GFET sensors with all characteristic I-V transfer curves recorded. For the laboratory investigation of the analytical performance of the GFET biosensor, MCF-7 exosomes were used as model exosomes, the working concentration of exosomes was prepared by 10-time serial dilution from the stock solution ((MCF-7 exosomes (Abcam, ab239691))), and × 1000 diluted PBS was used as the solvent. For clinical detection with PDAC patient and healthy control plasma, the transfer curves in ×1000 diluted PBS buffer solutions were recorded first. Then, the plasma sample (20 μL) was added to the functionalized sensor surface for 15 min of incubation, followed by a multiple wash step with PBS and DI water. Finally, the sensor immobilized with exosomes in plasma was tested in buffer. Total testing time is 45 mins that include the 15 mins incubation, multiple rinsing and 24 mins for two measurements.

3.7 Exosomes characterisation techniques

3.7.1 Nano-Flow Cytometry

Flow Cytometry is a promising technique for the studying of individual cells or microscopic particles. Conventional flow cytometry allows the characterising, counting, and sorting of cells or particles in the aqueous solution. The conventional flow cytometry is widely used for the characterisation of particles of several microns. In flow cytometry, suspended particles will flow pass single or multiple lasers in a hydrodynamically focused stream. The light scattering or fluorescent signals of particles will be recorded and analysed. It can provide information related to the size, morphology and shape of particles through light scattering measurements, and the biochemical information of biological samples through fluorescent labelling.

However, conventional flow cytometry has limited applications to the nanoscale extracellular vesicles (EVs). Nano-Flow Cytometry provides a solution to the limitations of conventional flow cytometry through several technical optimizations. Nano-Flow cytometry allows for size and concentration measurements of nanoparticles (7-1000 nm) isolated from biofluids. It is particularly useful in the detection of extracellular vesicles. The combination of light scattering and fluorescent detection allows the quick acquisition of high-resolution distribution of particle size and the biochemical properties of analysed particles. (Figure 3-15)

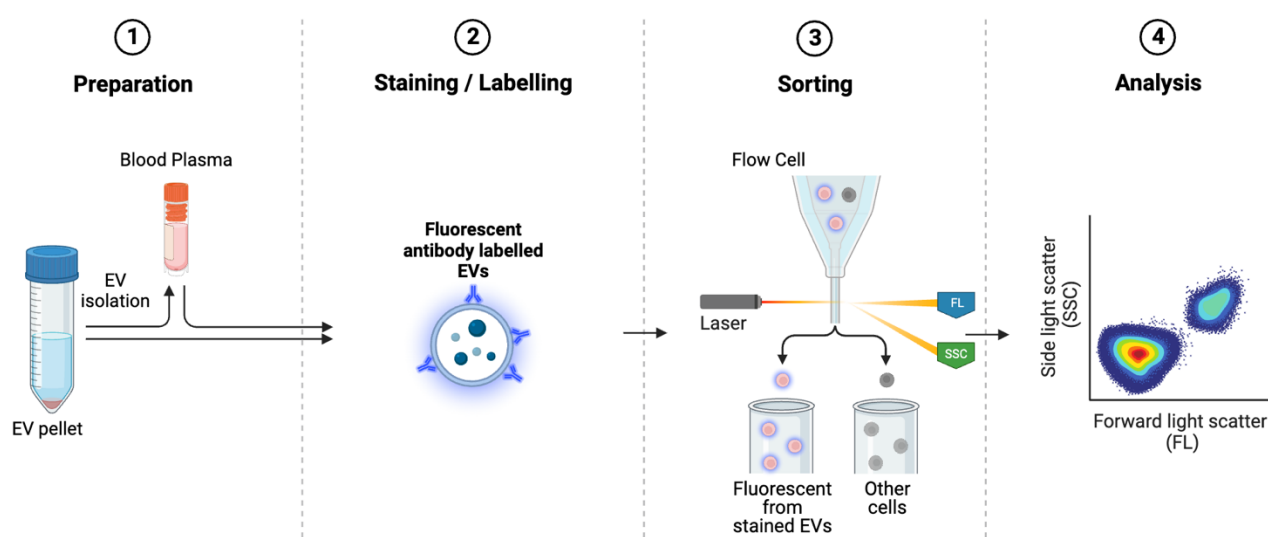


Figure 3-15 Schematic illustration of the flow cytometry analysis of EV samples. The combination of light scattering and fluorescent detection allows the quick acquisition of high-resolution distribution of particle size and the biochemical properties of analysed particles.

The presence of excess material in the solution may impact the light scattering event and introduce errors into EV measurements for Nano-Flow cytometry. Therefore, the samples need to be isolated from the biofluids prior to the analysis using Nano-Flow cytometry.

In order to achieve sensitive and accurate EV quantification and detection, the instrumental settings need to be optimized first. The best data is attained once flow cytometry workflows are optimized. The maintenance of high staining efficiency whilst reducing background fluorescence (FL) to keep a low LOD is crucial.

Plasma GPC-1+ exosome concentration and sizing were measured by Nano Flow cytometry using a NanoAnalyzer U30 instrument from NanoFCM Inc. (Nottingham, UK). First, 50 μ L of each blood plasma sample was incubated with 1 μ L of Alexa Fluor 647 conjugated anti-Glypican antibody (Abcam, ab237290) for 30 min at room temperature. After incubation, the mixture was diluted in PBS to 1 mL to pellet EVs, following by ultracentrifugation at 55,000 rpm (100,000 $\times$ g) with a benchtop optima TLX (Beckman Coulter) for 45 min. The supernatant was removed, and the pellet was resuspended in 50 μ L PBS. The re-suspended mixture was analysed in the Flow NanoAnalyser to determine concentration and fluorescence positivity. Data processing was performed using nFCM Professional Suite v1.8 software.

3.7.2 Exosome isolation from human plasma samples

Two different methods were used to isolate exosomes from human plasma samples, size exclusion chromatography (SEC) by Izon Column and ultracentrifugation. Both methods will be described in detail below.

3.7.2.1 Exosome isolation with Izon Column

The working principle of qEV columns are based on SEC for the separation of contaminating soluble protein from exosomes and other EVs. Izon qEV Original columns (70 nm separation) were used.

The Izon column was first taken from fridge and allow 15 minutes to reach room temperature (stored in Fridge at 4°C). The Izon column was set up on rack with labelled falcon tubes and eppendorfs. Then the sodium azide solution present in the column was discarded into a discard falcon tube by removing the top cap first before opening the bottom cap. The dropper cap was added to the column and 9 ml of PBS buffer were added to the top to equilibrate the column. The added PBS buffer was allowed to run through the Izon column into the discard falcon tube. When the PBS buffer process is completed, the discard falcon tube was replaced with a 15 ml void falcon tube. 1 ml of plasma sample was carefully added to the center of the Izon column to avoid any clinging to the wall of the Izon column. When the sample was loaded, i.e. no more samples remained above the filter, 15 ml of PBS buffer were loaded into the Izon column. The first 3 ml were collected in the void tube, then EV tube was used to collect the EV fraction. 2 mL of the isolated EV samples were collected. Samples were kept on ice until storage or further processing.

3.7.2.2 Exosome isolation using ultracentrifugation

For the isolation of exosome using ultracentrifugation, human plasma samples were centrifuged in a Beckman Coulter Optima XPN-80 Ultracentrifuge at $100,000 \times g$ at 4°C for 45 min, with a 50.2 Ti rotor (k-factor: 157.7) to pellet EVs. The supernatant was carefully removed, and the resulting pellets were resuspended in 50 µL PBS. The samples were further analysed or stored at -80°C.

3.7.3 Transmission Electron Microscopy (TEM) and Immunogold labelling

Transmission Electron Microscopy (TEM) is a high-resolution imaging technique, it works on the principle of transmission. In TEM, the image is formed through a beam of electrons passing through the thin samples. These electrons transmit through the sample and interact

with the sample, then fall on a fluorescent plate or camera detector, generating the image. TEM can achieve an extraordinary resolution, often below 0.1 nanometers (or 1 angstrom).

TEM can also be performed in conjunction with immunogold labelling. Immunogold labelling is a staining technique that utilizes the colloidal gold particles attached to secondary antibodies that are attached to primary antibodies. These primary antibodies are designed to bind with specific antigen or proteins. The immunogold labelling allows the visualization of specific proteins or antigens within the biological samples. Through targeting the specific surface biomarkers using the gold-conjugated antibodies, the precise identification, localization and quantification of specific proteins or markers on the surface of exosomes is enabled. The application of immunogold labelling with TEM is particularly useful in the imaging of exosomes.

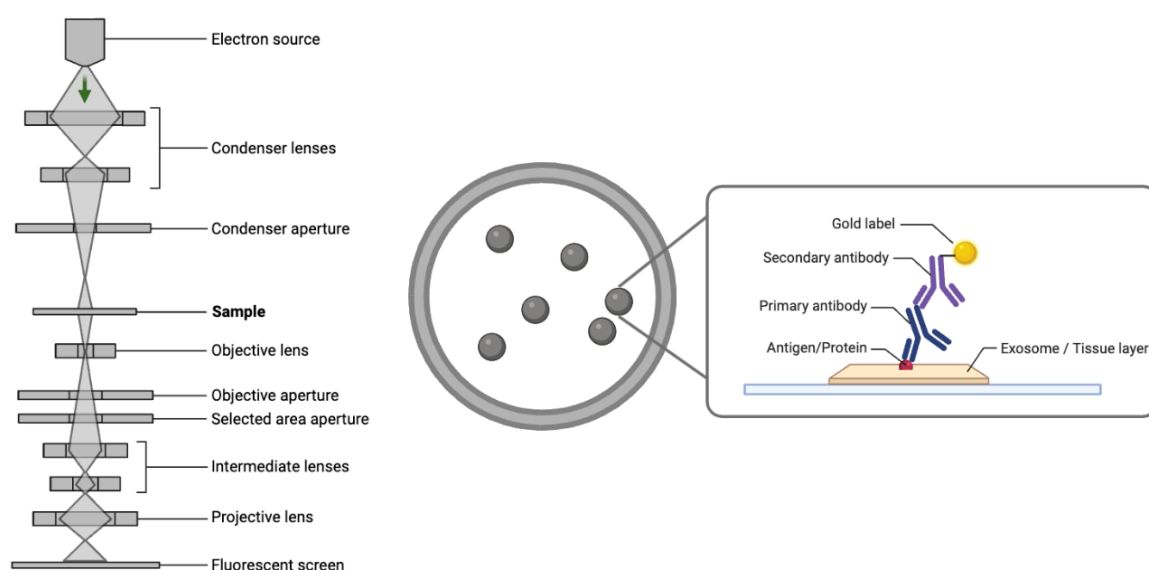


Figure 3-16 Transmission Electron Microscopy (TEM) and Immunogold labelling. A) The primary components of a TEM. B) Schematic representation of immunogold labelling mechanisms.

Transmission electron microscopy (TEM) characterisation was performed using a JEOL JEM-2100F field emission S/TEM equipped with an Oxford X-MaxN 80 mm² SDD detector for

elemental analysis. TEM images were acquired at 200 kV accelerating voltage and 116 μ A emission current. DigitalMicrograph GMS3 (Gatan) and Aztec TEM (Oxford Instruments) software packages were used for the TEM data and energy dispersive X-ray (EDX) spectra analysis and interpretation, respectively. Briefly, for the immuno-gold labeling with antibodies, GPC-1 antibodies (pa5-51290, Thermo Scientific) were attached on 10-nm gold particles (AURION) according to manufacturer's instructions at room temperature. Healthy plasma and PDAC plasma sample pools were prepared. 200 μ L of from each of 5 healthy or 5 PDAC plasma samples were taken to form a healthy plasma or PDAC plasma sample pool. Exosomes were isolated as previously described. Then isolated exosomes samples were conjugated with the gold-particles attached GPC-1 antibodies at the appropriate dilution overnight at 4°C. Samples for TEM characterisation were drop-cast from dilute aqueous suspensions onto amorphous carbon coated (200 nm) Cu grids (Agar Scientific) and dried naturally overnight.

3.7.4 Western blot analyses

Western blot analysis is a useful laboratory technique for the separation and detection of specific protein present in a blood sample. The western blotting can separate the mixture of proteins based on their molecular weight.

Frozen samples were first thaw on ice. Samples of isolated exosomes were concentrated first. Samples were added to concentration columns Vivaspin Turbo 4 with 10 kDa MW cutoff by spinning samples at 4000 $\times$ g for up to 2 hours at 4°C. Samples were checked every 30 minutes. Then the samples were lysed with Cell Lysis RIPA Buffer (10X) (#9803, Cell Signaling Technology) with the addition of PMSF (200mM, Cell Signalling Technology). The EVs samples were lysed with PMSF at 1:200 (final conc. 1mM) and RIPA 10x at a ratio of 1:10. The samples and lysis buffer were then vortexed for 20 second and cold lysis on ice for 30 minutes. Then the samples were centrifuged at 12,000 $\times$ g for 20 minutes. The supernatant was carefully removed and transferred into a new tube. Samples were then stored at -80°C or proceeded for further measurements.

The concentration of samples (1 μ l) were measured with Pierce™ BCA Protein Assay Kit (Thermo Scientific) in triplicate. Then the fresh 2M DTT solution was prepared by adding 0.3085g of DTT to 1000ml water. The Western blot analyses were then performed in DTT and Sample Buffer (Pierce™ LDS Sample Buffer, Non-Reducing (4×), Thermo Scientific) using the selected antibodies: 1:500 ALIX (#92880, Cell Signalling Technologies), 1:250 Glypican-1 (PA5-24972, Thermo Scientific), CD9 (sc-13118, Santa Cruz, mouse), CD63 (MX-49.129.5, Santa Cruz).

3.7.5 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) was selected for the analysis of the size distribution of the isolated exosomes. The principal of DLS for the determination of particle size is based on the measurement of the random changes in the intensity of light scattered from the suspension or solution. The small particles in the suspension undergo random thermal motion, known as the Brownian motion. These particles move random in the solution, having continuous collision with surrounding solvent molecules and cause intensity fluctuations of scattered light. The smaller the particle, the faster it moves. These fluctuations are measured and used to calculate the relative size distribution of particles.

Dynamic light-scattering measurements were performed using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). The intensity, volume, and distribution data for each sample were collected on a continuous basis for 4 minutes in sets of three. Three different measurements from the isolated exosome samples were performed.

3.8 Density functional theory (DFT) Simulation

The density functional theory (DFT) is a computational quantum mechanical modelling method used to investigate the geometric and electronic structure of many-body systems, in particular atoms and molecules. DFT is based on the concept of electron probability density. Using DFT, the properties of the many-electron system can be obtained using the functionals.

The use of DFT calculations allow the prediction and calculation of material behaviour without the need of higher-order parameters.

A theoretical simulation based on density functional theory (DFT) has been performed to interpret how the linker molecule PBASE or TCPP adsorbed on graphene using the VASP package.[231] Generalized Gradient Approximation (GGA)-Perdew-Burke-Ernzerhof (PBE) was used to improve the exchange-correlation energy description.[232] The projector augmented wave (PAW) method was implemented to effectively describe the electronic structure of the full valence and core wave.[233] A cut-off energy of 400 eV was set for the plane wave under periodical boundary conditions. A 1*1*1 Gamma centred mesh was used to sample Brillouin Zone. Geometry optimization based on an 8*8*1 supercell was performed with energy converge criteria of 10^{-6} eV and force converge criteria of 0.01 eV/Å. For molecule adsorption onto 2D material, Grimme's DFT_D3 method was used to describe a dispersion interaction in order to accomplish a van der Waals (vdW) correction.[234], [235]

3.9 Statistical analysis

For all experiments, quantitative results are presented as mean $\pm$ standard deviation (s.d.), where n denotes the number of replications.

The statistical significance of the data was assessed using the two-sample Student's t-test and is designated with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ **** $P < 0.0001$).

4 Probing the Orientation of Antibodies on graphene biosensor

4.1 Key Novelty and Achievements

The past decade has seen researchers develop and apply graphene technologies in biomarkers detection due to their advantages of real-time, label-free detection and high sensitivity. At the heart of these technologies is the immobilization of antibodies onto the graphene surface using noncovalent linker molecules. Yet, the critical role of these linker molecules in determining antibody orientation, and subsequently, the performance of graphene biosensors, has been largely uncharted.

In order to enhance the performance of GFET biosensors with high capturing efficiency of target biomarkers on graphene, a new biofunctionalization protocol using tetrakis (4-carboxyphenyl) porphyrin (TCPP) as the linker molecule is developed and employed. This Chapter aims to address this crucial aspect in graphene biosensors by probing the orientation of antibodies on graphene surface functionalised using two different linker molecules, tetrakis (4-carboxyphenyl) porphyrin (TCPP) and 1-pyrenebutanoic acid succinimidyl ester (PBASE). With the newly developed TCPP method, it is demonstrated that this approach can refine the orientation of conjugated antibodies on graphene surfaces. Through rigorous analyses employing Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) and Density-functional theory (DFT) simulations, the predominant end-on vertical orientation of the Immunoglobulin G (IgG) antibody on TCPP-functionalized graphene, as opposed to the varied orientations on PBASE-functionalized graphene was identified.

The work presented in this Chapter is part of the Journal publication: Tianyi et al. Control of antibody orientation on graphene using porphyrin linker molecules for high-performance graphene-based immuno-biosensors,” Submitted.

DFT simulation was done in collaboration with Xu Xi. The portable electronic device was designed by Dr. Bruno Gil Rosa.

4.2 Overview

GFET biosensors have been deployed for the detection of a variety of biological species and disease biomarkers, including nucleic acids,[148] proteins,[193], [236] dopamine,[152] extracellular vesicles,[68], [86] cancer biomarkers,[20] pathogenic viruses,[4], [237, p. 19] and other disease biomarkers.[61], [218]

In such applications, the graphene surface is functionalized with linker molecules that have dual functionality: to stack on graphene and, at the same time, to form strong bonds with biological probes such as antibodies, DNAs, and aptamers. The most widely used linker molecules for graphene-based biosensors are pyrene-based ligands, including 1-pyrenebutanoic acid succinimidyl ester (PBASE),[63], [68], [86], [193], [230] 1-pyrenebutyric acid (PBA),[63], [238] and pyrene-propanoic amid ethylmaleimide (PMAL)[63], [238]. These pyrene linkers can form non-covalent functionalization onto graphene surface and covalent bonds with antibodies. In addition, their small size and self-limiting nature make them suitable for electrical detection, where Debye screening is a major issue in biosensor performance. Porphyrin-based ligands are a family of planar and macrocyclic compounds with a characteristic tetrapyrrole structure which are another type of linker molecule for graphene-based biosensors. Porphyrins have been extensively studied due to their excellent photoelectrical and catalytic performance. In recent years, porphyrins have been used with graphene oxides as hybrids for photocatalytic activity,[239], [240] photovoltaics,[241] optoelectronics,[242] photoelectrochemical sensing[243], [244], [245], and biosensing.[103], [104] The use of members of the porphyrin family as linker molecules for graphene could potentially enhance the sensing performance of graphene-based biosensors. The aromatic porphyrin group attach to graphene through π - π stacking, and the remaining -COOH group can be readily modified using EDC/NHS (N-ethyl-N'-(3-(dimethylamino)propyl)carbodiimide/N-hydroxysuccinimide) chemistry for the covalent attachment of antibodies. The stack of linker-graphene can play a crucial role in the formation and orientation of antibodies on graphene and the sensitivity and limit of detection of GFET biosensors. In studies of these biosensors, it is assumed that conjugated antibodies are immobilized upright on the linker molecules on the graphene surface due to the strong binding between graphene and aromatic groups via π - π interaction. Yet there are very few

reports that investigate the role of the linker molecules on the orientation of antibodies on graphene surfaces and its impact on the sensitivity of graphene biosensors.[138]

This chapter aims to investigate the properties of the functionalisation process with different linker molecules, tetrakis (4-carboxyphenyl) porphyrin (TCPP) and 1-pyrenebutanoic acid succinimidyl ester (PBASE). The antibody orientation on the functionalized graphene surface is probed.

4.3 Characterisation of graphene functionalized with TCPP and PBASE linkers

A schematic of surface-immobilized antibodies with different possible orientations on the graphene biosensor is demonstrated in Figure 4-1. The antibodies immobilized on the surface of graphene in this study are conjugated using two different graphene functionalization linker molecules: PBASE and TCPP. For PBASE-functionalized graphene biosensors, antibodies are covalently attached to the N-hydroxysuccinimide (NHS) ester “end” on the highly flexible alkyl chain. It is expected that the antibodies will have multiple orientations on the graphene surface, including lying flat, vertical, or tilted at different angles. In contrast, antibodies immobilized on graphene biosensors functionalized with TCPP are expected to covalently bind to the NHS ester bond on EDC/NHS-activated bulky TCPP molecules lying flat on the graphene, thereby forcing the formation of the primary amide bond at a reduced tilt angle or even a vertical perpendicular orientation with respect to the graphene surface.

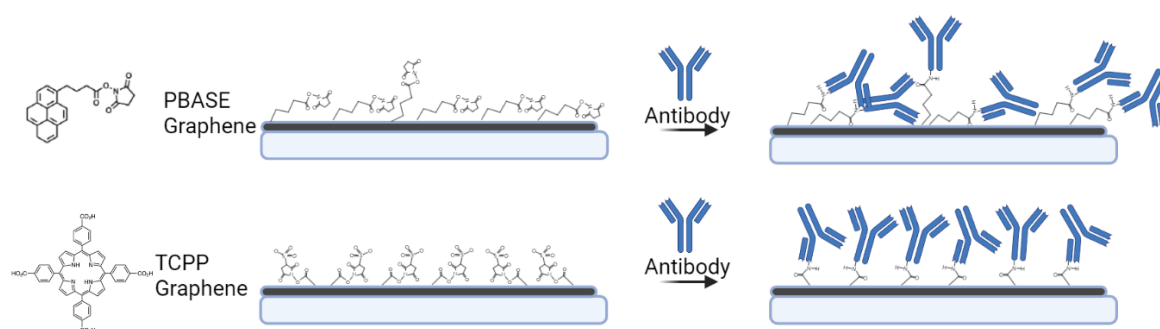


Figure 4-1 Schematic diagram of surface-immobilized antibodies with different possible orientations on graphene biosensors functionalized using PBASE and TCPP as linker molecules.

Various characterization techniques were performed to examine the functionalization of linker molecules and antibodies on the graphene surface.

4.3.1 Raman Spectroscopy

The influence of functionalization with PBASE and TCPP on the properties of graphene was investigated using Raman spectroscopy.

A comparison of Raman spectra recorded on bare graphene and graphene after functionalization is shown in Figure 4-2. The Raman spectra recorded for bare graphene showed peaks at 1580 cm^{-1} , which is commonly attributed to the G band of sp^2 carbon in graphene. In addition, the presence of a peak at 2700 cm^{-1} (2D secondary D band) is also shown, with another small intensity peak at 1350 cm^{-1} (D band), indicating a high quality of monolayer graphene. Raman spectroscopy analysis after functionalization with PBASE demonstrated a strong increase in the disorder D peak at 1350 cm^{-1} , which is due to the resonance of sp^3 bonding or to localized vibrational modes of the PBASE interacting with extended phonon modes of graphene.[246], [247] Raman spectra of the graphene surface after modification with TCPP showed the characteristic peaks of TCPP, which are vibrations of C-N (1240 cm^{-1}), C-H (1454 cm^{-1}), C-C (1565 cm^{-1}), and a weak shoulder peak (1496 cm^{-1}). [248]

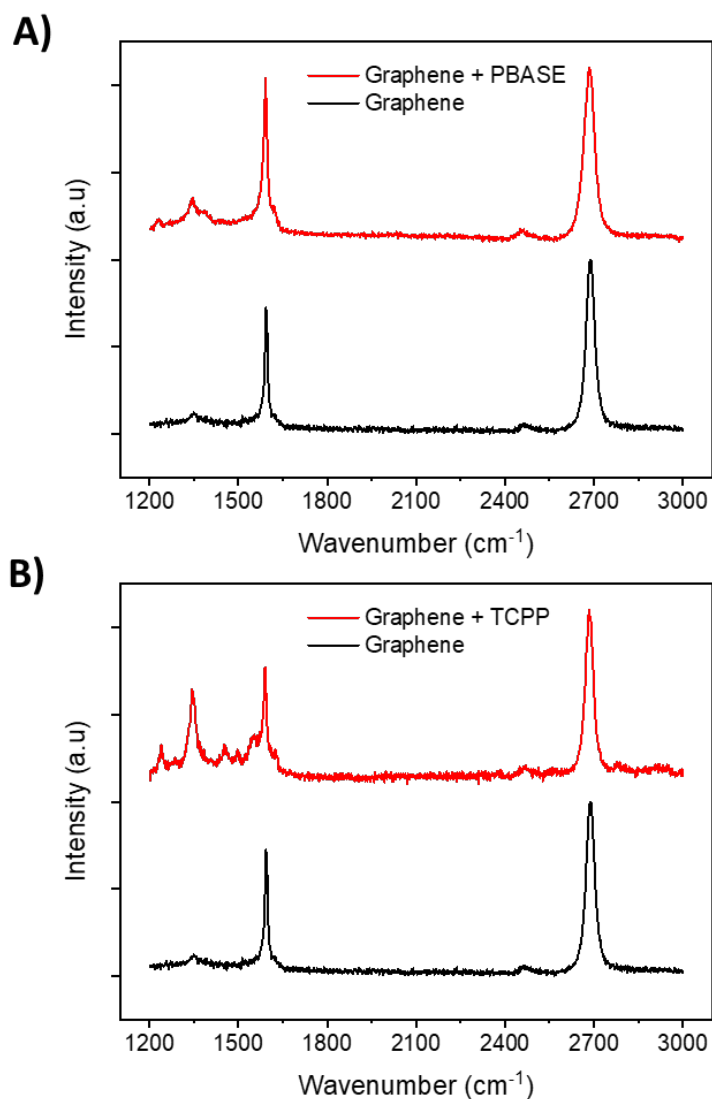


Figure 4-2 A) Raman spectra of bare graphene and PBASE-functionalized graphene. (B) Raman spectra of bare graphene and TCPP-functionalized graphene.

Further, Raman was employed to map graphene surfaces functionalized with PBSAE and TCPP, both before and after antibody immobilization, to understand the secondary and tertiary structures of proteins better. For antibodies, the Raman spectrum displays peaks corresponding to various molecular vibrations associated with the protein and any bound ligands. Tryptophan residues, which often show peaks around 760 cm⁻¹ and 1550 cm⁻¹. Tyrosine residues with characteristic shifts around 830 cm⁻¹. The Amide III band, ranging from 1200-1300 cm⁻¹, arises from a combination of N-H bending and C-N stretching vibrations and reflects the protein's secondary structure. Raman bands for the amino side chains of proteins

are primarily found between 700 and 1000 cm^{-1} . The Amide I band, located at $\sim 1650 \text{ cm}^{-1}$, primarily results from the C=O stretching vibration of the peptide backbone and is sensitive to the protein's secondary structure. Different secondary structures, such as alpha-helices, beta-sheets, and random coils, produce slightly varied peak positions within this region. [249], [250], [251], [252]

To further confirm the immobilization of the antibody on the graphene surfaces, samples were mapped for peaks at 760, 830, 1450, 1550-1560, and 1650-1660 cm^{-1} , as illustrated in Figure 4-3. Additionally, the graphene functionalized with TCPP and PBASE was characterized using Raman spectroscopy, as depicted in Figure 4-4.

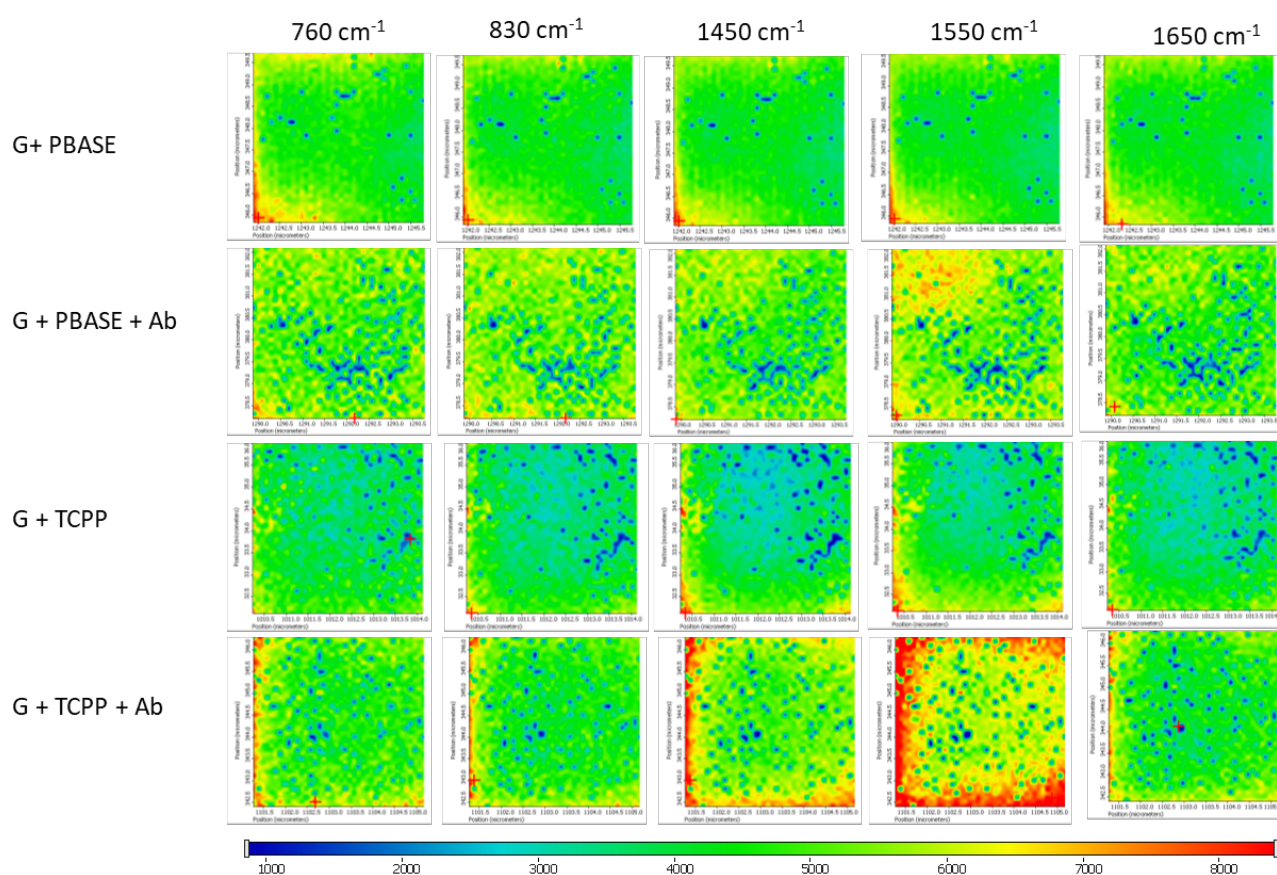


Figure 4-3 Raman mapping of graphene surfaces functionalized with PBSAE and TCPP before and after immobilisation with antibody. Raman mapping experiment was conducted over a $4 \times 4 \mu\text{m}^2$ area of graphene with PBASE and TCPP, both with and without antibodies.

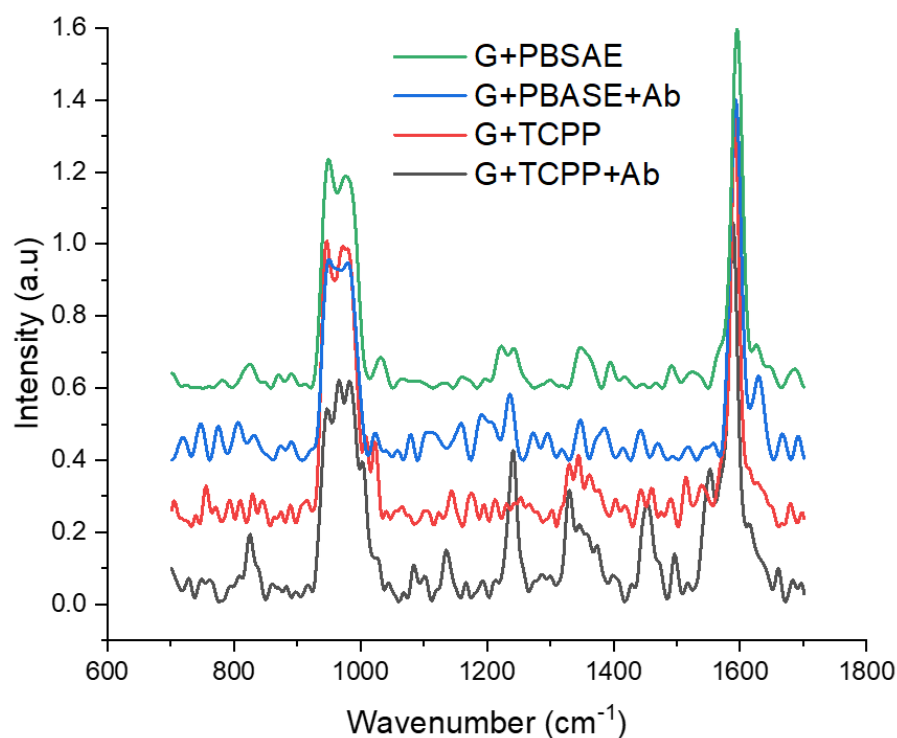


Figure 4-4 Raman spectra of graphene functionalized with TCPP and PBASE before and after immobilization with antibodies.

4.3.2 AFM

Atomic force microscopy (AFM) was used to validate the presence of the linker molecules of PBASE or TCPP on the surface of graphene (Figure 4-5). The surface roughness of pristine graphene was measured at 0.30 nm. After functionalization with PBASE for 2h, the surface roughness increased to 0.44 nm, while with TCPP treatment, the surface roughness of the graphene increased to 0.49 nm. Specific antibodies (anti-CD63 antibodies) were then immobilized on the linker molecule-functionalized surface. Both PBASE- and TCPP-functionalized graphene showed good surface coverage of antibodies, with increases in surface roughness to 0.80 nm and 1.02 nm respectively.

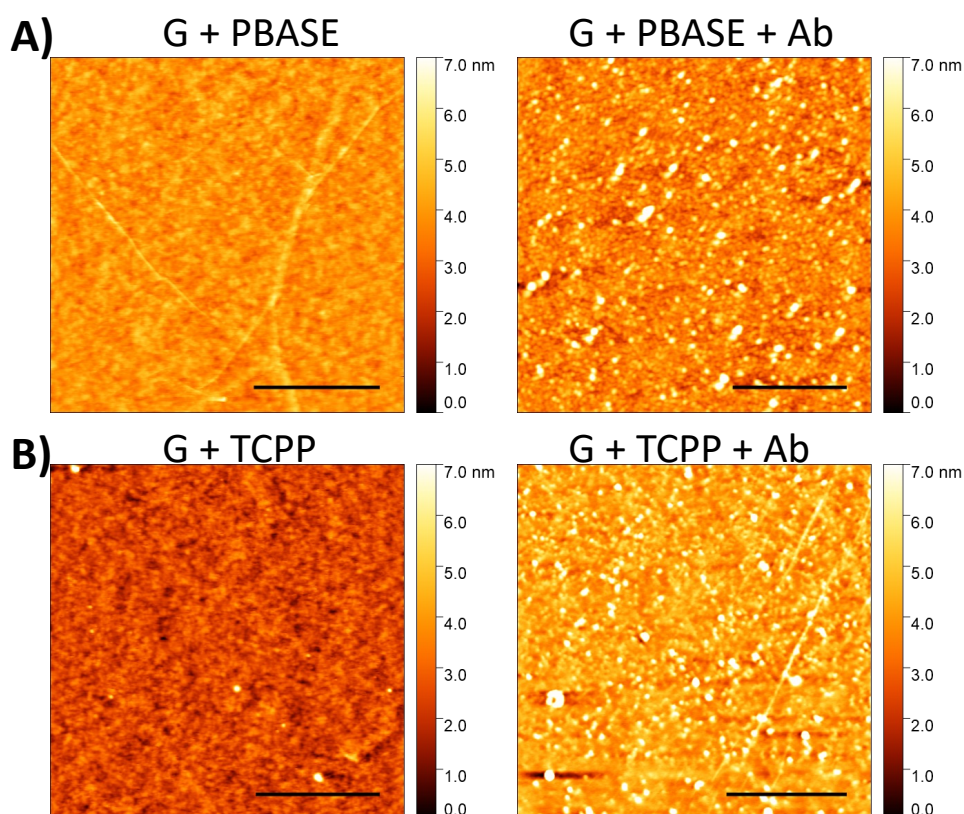


Figure 4-5 Atomic force microscopy (AFM) profile for characterization of: (A) PBASE-modified graphene (surface roughness = 0.44 nm), graphene surface after PBASE + antibody biofunctionalization (surface roughness = 0.80 nm); (B) TCPP-modified graphene (surface roughness = 0.49 nm), and graphene surface after TCPP + antibody biofunctionalization (surface roughness = 1.02 nm). AFM scale bars = 1 μ m.

4.3.3 XPS

X-ray photoelectron spectroscopy (XPS) was used to confirm the presence of PBASE or TCPP and antibody on the graphene surface. Figure 4-6 and Figure 4-7 show the changes in principle C 1s and N 1s core levels after each functionalization step.

The high-resolution N 1s spectra show a significant increase in the N 1s peak at 399.8 eV after GPC-1 antibody conjugation. (Figure 4-6) This N 1s peak is attributed to the successful formation of amide bond (CONH) between antibody and the linker molecules and large group of amines present on the antibodies.

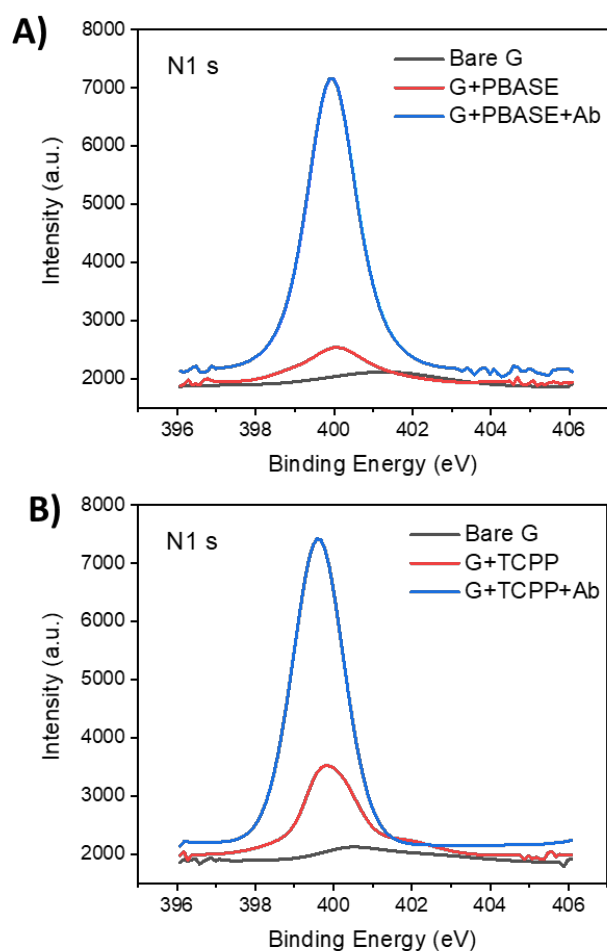


Figure 4-6 N1 s of X-ray photoelectron spectroscopy (XPS) spectra of graphene with different degrees of functionalization: (A) bare, with PBASE, and with PBASE + antibody respectively; (B) bare, with TCPP, and with TCPP + antibody respectively.

The high-resolution C 1s spectrum is composed of four components at the surface, C–C at 284.9 eV, C–O at 285.9 eV, C–N at 286.4 eV, and O–C=O at 287.7 eV, corresponding to a large number of amine and amide bonds present in the antibodies. (Figure 4-7)

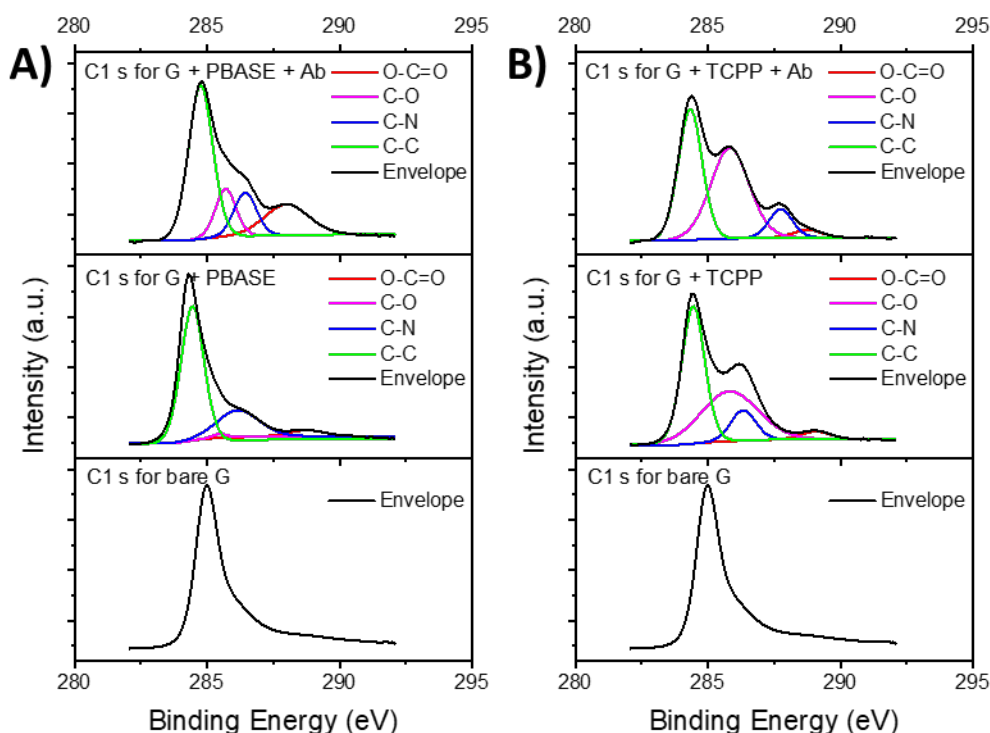


Figure 4-7 C1 s of X-ray photoelectron spectroscopy (XPS) spectra of graphene with different degrees of functionalization: (A) bare, with PBASE, and with PBASE + antibody respectively; (B) bare, with TCPP, and with TCPP + antibody respectively.

4.3.4 Water contact angle (WCA) measurements

The water contact angle (WCA) measurements were performed to study the influence of the linker molecule PBASE or TCPP and antibody on the graphene surface to the hydrophobicity of the graphene surface, as demonstrated in Table 4-1 and Figure 4-.

The WCA of bare SiO₂/Si substrate and graphene on SiO₂/Si substrate is $68.9 \pm 0.8^\circ$ and $83.7 \pm 0.4^\circ$, respectively. There is an increase in WCA with graphene on the substrate, indicating an increase in hydrophobicity after graphene transfer. This is in good agreement with the results reported in the literatures, the nature of graphene is slightly hydrophobic.

After the functionalization with linker molecules, both graphene with PBASE and TCPP, the surface of graphene demonstrated a reduction in its WCA. The reduction in WCA for both PBASE and TCPP functionalized graphene is due to the ester groups on these linker molecules

facing out of the surface of graphene.[253] This suggests successful functionalization of linker molecules, PBASE or TCPP, on the surface of graphene.

Table 4-1 Summary of water contact angle of SiO₂/Si substrates with and without graphene and after surface functionalization with linker molecules. Data are mean $\pm$ standard deviation (s.d.). n = 5 for each measurements.

Substrate	Water Contact Angle / °
SiO ₂ /Si	68.9 $\pm$ 0.8
G/ SiO ₂ /Si	83.7 $\pm$ 0.4
PBASE/G/SiO ₂ /Si	59.7 $\pm$ 1.0
TCPP/G/SiO ₂ /Si	60.1 $\pm$ 0.9

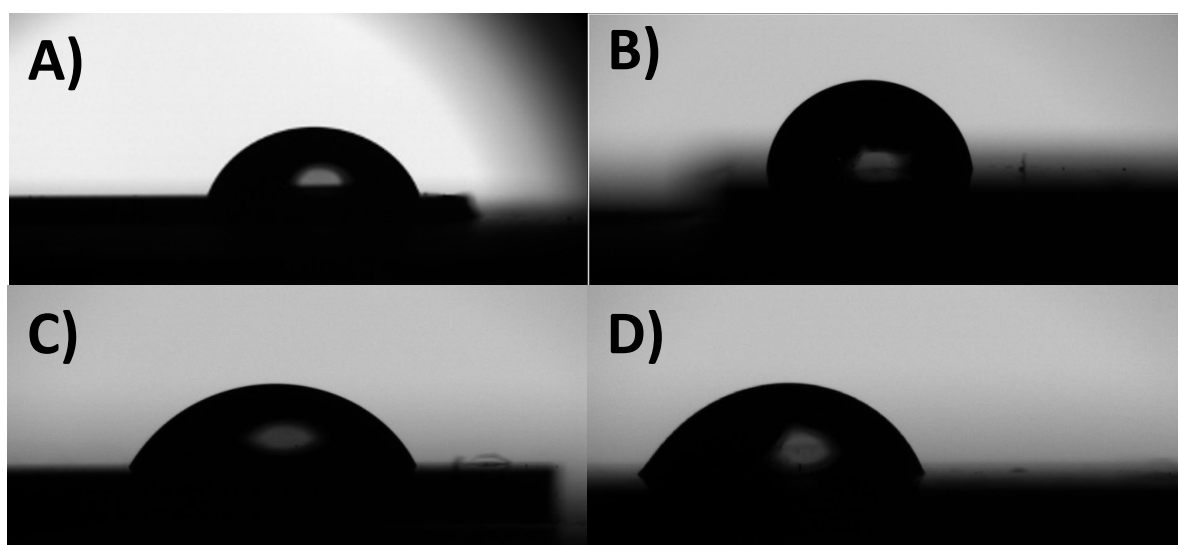


Figure 4-8 Images of WCA measurements of SiO₂/Si substrates with and without graphene and after surface functionalization with linker molecules. WCA measurements of A) SiO₂/Si, B) G/SiO₂/Si, C) PBASE/G/SiO₂/Si, D) TCPP/G/SiO₂/Si.

4.4 Characterisation of antibody orientation on graphene surface with TOF-SIMS

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) was employed to characterize proteins and reveal differences in antibody orientation on the functionalized graphene surfaces.[254], [255], [256], [257], [258], [259] TOF-SIMS is a powerful technique in revealing the chemical information of the surface of the materials. Leveraging the characteristic ion fragments derived from the amino acid composition of various proteins, TOF-SIMS analysis can determine the orientation of antibodies on substrates. [254], [255], [260], [261] Given the intricate nature of the data produced by TOF-SIMS, principal component analysis (PCA)[262] was utilized to enhance the sensitivity in differentiating between highly similar surface compositional changes associated with proteins.

A detailed description of the principle underlying the PCA analysis of TOF-SIMS data for antibody orientation characterization is provided in the sections below. The PCA model was formulated using the TOF-SIMS data collected for samples of TCPP- and PBASE-functionalized graphene substrates, as well as from the functionalized substrates with immobilized $F(ab')_2$, Fc fragments and IgG antibodies. To examine the surface orientation of adsorbed IgG antibodies, the TOF-SIMS data collected for functionalized graphene with immobilized antibodies were projected onto the developed PCA model (Figure 4-9).

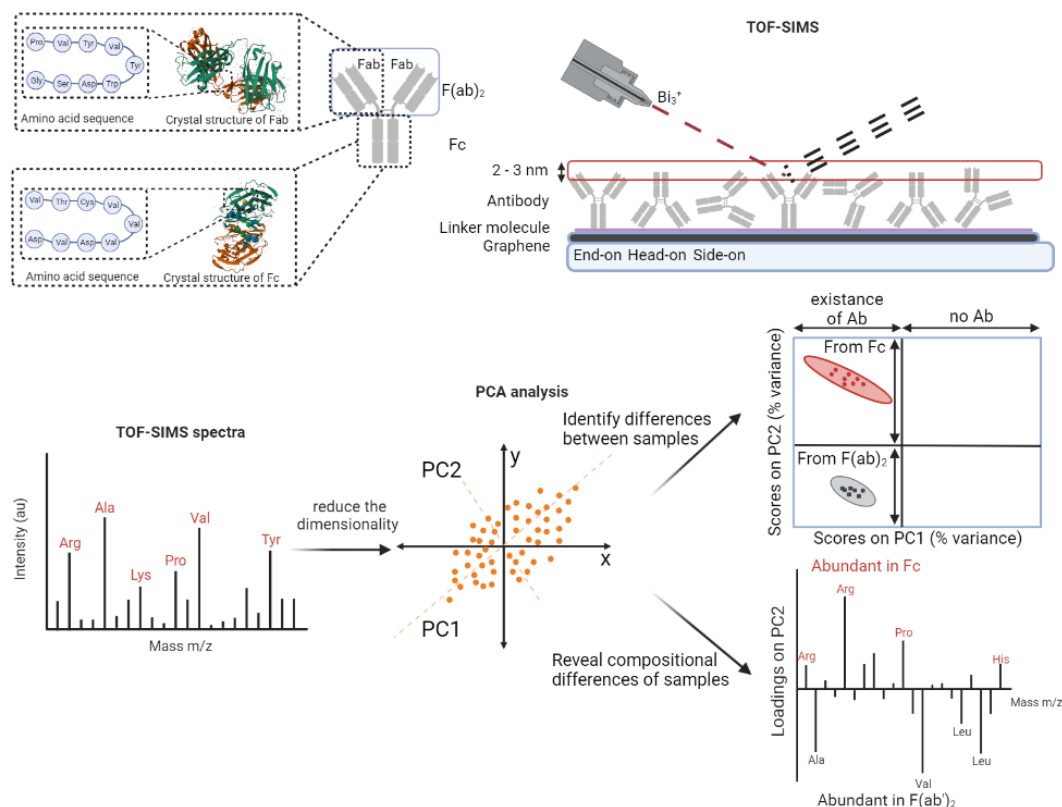


Figure 4-9 Schematic diagram for SIMS with PCA for antibody orientation characterization. In SIMS, three different antibody fragments were chosen for the analysis of orientation. A well-studied IgG antibody with known amino acid sequence was chosen.

4.4.1 Antibodies

Antibodies are composed of two heavy chains and two light chains which are folded into a characteristic Y-shape. (Figure 4-10) The tips of the Y-shaped molecule contain the fragment antigen-binding regions (Fab) which recognize and bind to the target analyte. The two antigen-binding F(ab) regions are linked together by disulphide bonds and form the F(ab')₂ fragment. The fragment crystallizable region (Fc) located at the base of the Y-shaped molecule is the constant, crystallizable portion of the antibody molecule.

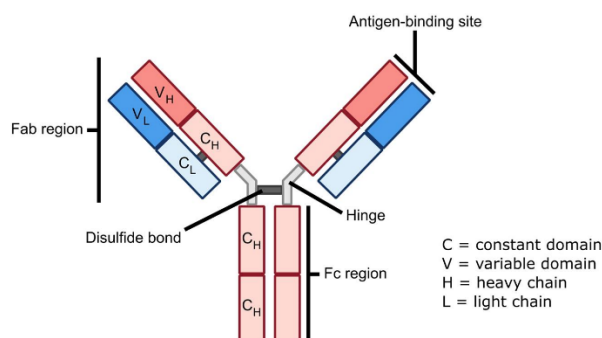


Figure 4-10 Architecture of an antibody. Antibodies are composed of two heavy chains and two light chains which are folded into a characteristic Y-shape. The tips of the Y-shaped molecule contain the fragment antigen-binding regions (Fab); The fragment crystallizable region (Fc) located at the base of the Y-shaped molecule.

4.4.2 Principal Component Analysis (PCA)

Multivariate analysis is a statistical analysis technique that allows the simultaneous analysis of multiple variables. Multivariate analysis is very useful in determining the relationship or dependence between different variables. The application of multivariate analysis is particularly useful in analysing and simplifying complex data sets involving a large number of dependent variables. Multivariate analysis aided the data processing of complexed and multicomponent surfaces such as biosensors and biological materials.

There are many different multivariate analysis methods available for the exploration and elucidation of different features of the data, including multivariate curve resolution (MCR), neural networks (NN), principal components analysis (PCA), etc.. Among all of these multivariate analysis methods, PCA is the most commonly employed method for the processing of TOF-SIMS data. PCA can reduce the matrices of data into lower dimensionality through the description of these data using a small number of orthogonal factors. PCA can identify the major directions of variation present in the dataset. During the PCA analysis, the data set is defined as a matrix, with rows representing samples and columns representing variables.

A schematic diagram illustrating the main steps in principal component analysis (PCA) is shown in Figure 4-11. The first step of PCA is to standardize the data. This standardization step is performed to ensure the range of the continuous variables of the dataset is standardized to unit variance to avoid bias. Then, the covariance matrix is calculated. This covariance matrix will provide information regarding the correlation between the variables of the datasets. When the sign of the covariance is positive, it indicates the two variables are correlated. On the contrary, when the sign of the covariance is negative, it indicates the two variables are inversely correlated. Then eigenvalues and corresponding eigenvectors of this covariance matrix is calculated to determine the principal components of this dataset. The first principal component describes the direction of the largest variance of the data points in the data set. The second principal component is uncorrelated with (i.e., perpendicular to) the first principal component, and the second principal component accounts for the next highest variance.

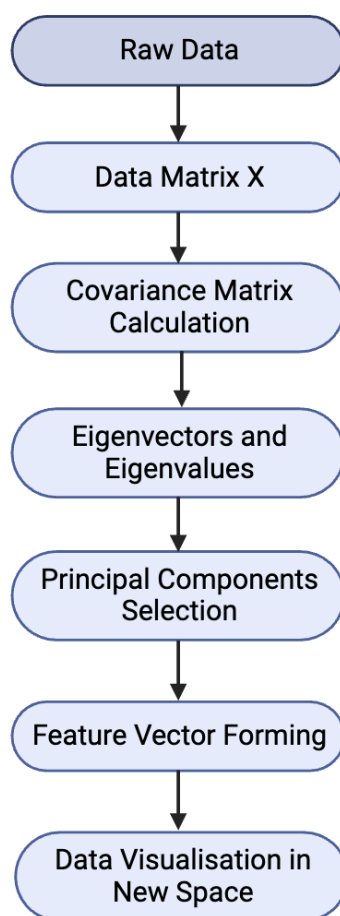


Figure 4-11 Schematic diagram of main steps in principal component analysis (PCA).

PCA decomposes the complex data matrix into matrices of scores and loadings. The scores for the principal components reveal the similarities or differences between samples, and the corresponding loadings show how strongly each characteristic influences the principal component, revealing the compositional differences among samples. The variance of each principal component reflects both intergroup dissimilarities and the dispersion of data points within each group.

4.4.3 Sample preparation and graphene functionalisation for TOF-SIMS measurement

The whole IgG antibody, its $F(ab')_2$ and Fc fragments contain the same amino acids and peptide sequences, only very few chemical species can represent the differences of these fragments. The $F(ab')_2$ and Fc fragments were used as the reference for the interpenetration of the orientation of IgG antibodies on the surface of graphene.

In order to study the orientation of immobilized antibodies on the functionalized graphene surface, an IgG antibody with a known amino acid sequence, along with its corresponding $F(ab')_2$ and Fc fragments need to be used in combination with the TOF-SIMS analysis.

First the graphene substrates were cut into 1 cm × 1 cm squares and functionalized with the TCPP or PBASE method. Then, the selected whole antibody IgG and its corresponding $F(ab)_2$ and Fc fragments were immobilized onto the surface of each separated functionalized graphene substrates and incubated overnight at 4 °C. The samples were then further rinsed with 1 × PBS and DI water to remove the excess unbind protein and salt. The prepared samples were immediately used for TOF-SIMS analysis or stored at 4°C for later use.

4.4.4 TOF-SIMS and PCA Analysis

TOF-SIMS is a highly sensitive mass spectroscopy technique for surface characterization. It provides the elemental and molecular surface chemical information through focusing a

primary ion beam onto the sample and analysing the secondary ions emitted from the surface. More detail of TOF-SIMS analysis is introduced in section 3.2.9. A typical spectrum data generated by TOF-SIMS contain hundreds of peaks, the peak intensity varied for different composition, structure and the orientation of different species on the surface. Manual comparison of the differences of the mass spectra between each sample is impossible. It is challenging to determine the correlation between peaks and their relation to the chemical composition difference on the surface. A large dataset is generated from the TOF-SIMS technique and needs proper analysis. Therefore, PCA is useful in processing the complex datasets generated from TOF-SIMS.

To reveal differences in antibody orientation on the functionalized graphene surfaces, multivariate PCA analysis was employed for the analysis of the TOF-SIMS data (Figure 4-12). TOF-SIMS analysis was used to characterize proteins adsorbed on graphene substrates (Figure 4-12). However, due to the complexities of TOF-SIMS data, the multivariate statistical analysis technique of principal component analysis (PCA)[262] was used to reduce the dimensionality of TOF-SIMS data while retaining the essential information. PCA analysis can provide increased sensitivity for TOF-SIMS data in distinguishing between highly similar surface compositional changes in proteins.

For the TOF-SIMS analysis aimed at investigating antibody orientation on the graphene surfaces, we selected an IgG antibody (polyclonal goat anti-rabbit IgG) with a known amino acid sequence, along with its corresponding F(ab')₂ [263] and Fc [264] fragments. A set of 36 positive ion fragments with known mass spectra for amino acids[254], [265] was examined to interpret the subtle differences in the outermost surface composition of antibody adsorbed on the two functionalized graphene surfaces (Table 4-2).

Table 4-2 Characteristic positive ion amino acid fragments and their molar masses used for the TOF-SIMS analysis.

Ion fragment	Molar mass	Source of amino acid
C ₂ H ₆ N ⁺	44.05	Ala / lys
C ₃ H ₆ NO ⁺	72.05	Ala / Thr

CH_2N+	28.02	Arg
CH_3N_2+	43.03	Arg
C_4H_8N+	70.07	Arg / Pro / Val
C_2H_7N_3+	73.06	Arg
CH_2NO+	44.01	Asn
C_3H_4NO+	70.03	Asn
C_3H_7N_2O+	97.06	Asn
C_3H_6NO_2+	98.02	Asp
CHS+	45	Cys
C_4H_6NO+	84.05	Gln / Glu
C_4H_8NO_2+	102.06	Glu / Thr
CH_4N+	30.03	Gly
C_4H_5N_2+	81.05	His
C_5H_8N_3+	110.07	His
C_5H_10N+	84.08	Ile / Leu / Lys
C_5H_12N+	86.1	Ile / Leu
C_5H_13N+	87.11	Ile / Leu
C_2H_7N+	45.06	Lys
C_3H_6N+	56.05	Lys
C_2H_5S	61.01	Met
C_5H_9SO+	117.04	Met
C_8H_10N+	120.08	Phe
C_9H_7O+	131.05	Phe
C_4H_6N+	68.05	Pro

C_5H_6N+	80.05	Pro
C_2H_6NO+	60.05	Ser
C_3H_3O_2+	71.01	Ser
C_3H_8NO+	74.06	Thr
C_9H_8N+	130.07	Trp
C_11H_8NO+	170.06	Trp
C_7H_7O+	107.05	Tyr
C_5H_7O+	83.05	Val
C_4H_10N+	72.08	Val
C_4H_4NO_2+	98.02	Asn
CH_5N_3	59.05	Arg
C_10H_11N	159.09	Trp
C_9H_7O_2+	147.04	Tyr
C_8H_10NO	136.07	Tyr

Examples of positive ion TOF-SIMS spectra of the adsorbed IgG, F(ab)₂, and Fc on PBASE-functionalized and TCPP- TCPP-functionalized graphene are shown in Figure 4-12. For the analysis of TOF-SIMS data with PCA, the samples are the different TOF-SIMS mass spectra obtained from different samples, the variables are the measured intensities of each individual mass channels or the integrated peak areas of selected masses (m/z).

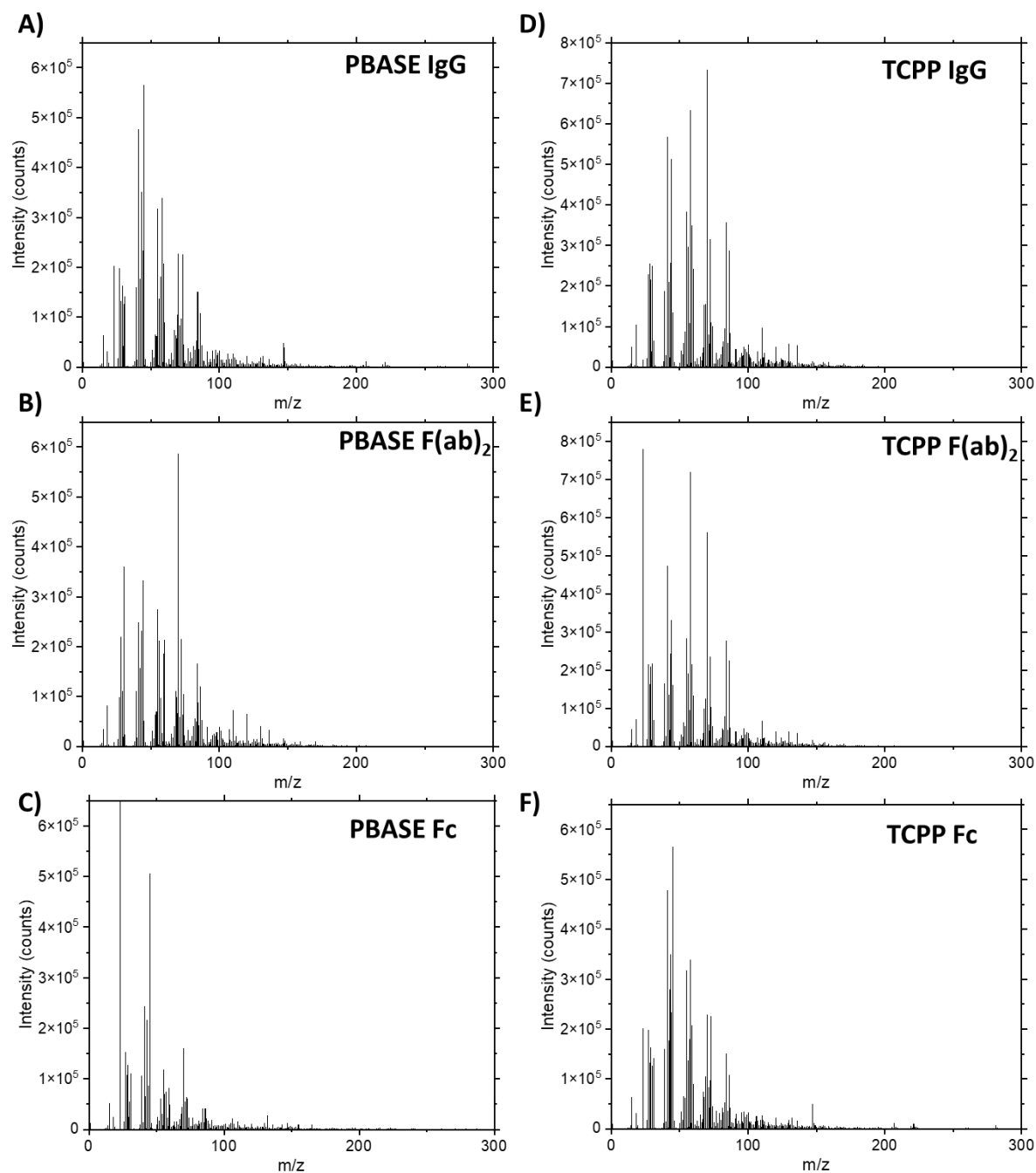


Figure 4-12 Example positive ion TOF-SIMS spectra of adsorbed IgG, F(ab)₂, and Fc on PBASE-functionalized and TCPP- functionalized graphene.

Principal component analysis (PCA) was carried out for a multivariate analysis (MVA). For the PCA analysis, only mass spectral peaks with a maximum intensity greater than 10^2 were selected to construct the matrix. Prior to PCA, the intensities of all selected TOF-SIMS peaks were normalized to their total intensities and then mean-centred. PCA was performed using

sklearn (Python library).[266] The PCA model was constructed using data from the reference sample of adsorbed F(ab')₂ fragments. The data from adsorbed Fc fragments and IgG on different functionalized substrates were projected onto the constructed PCA model to obtain their scores.

4.4.5 Discussion

The PCA results revealed that the first principal component, PC1, captures 55.06% of the total variance in the data set, while PC2 captures 40.73% of the variance which is not correlated with that described by PC1, and PC3 (2.45%) and PC4 (0.01%) appear not to be informative. The score plot of PC1 against PC2 for the PCA model developed for the functionalized graphene substrates is shown in Figure 4-13. It can be seen that PC1 shows good separation between the presence and the absence of the protein on the graphene surface. The PC2 score plot can clearly distinguish between the difference in amino acids located at various groups of proteins immobilized on the graphene surface. Substrates immobilized with F(ab')₂ fragments loaded negatively to PC2, whereas substrates immobilized with Fc fragments loaded positively.

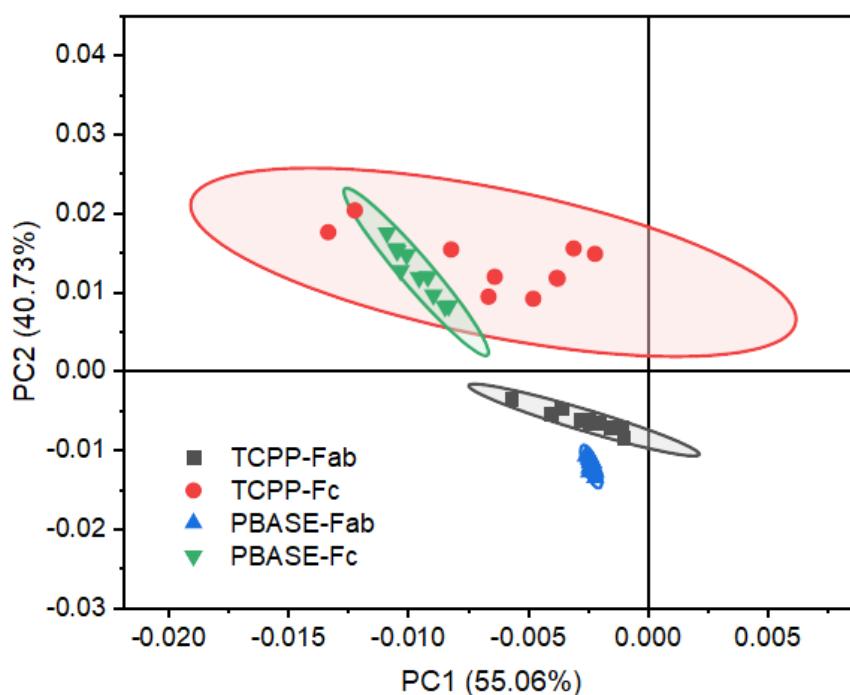


Figure 4-13 Principal component analysis of F(ab')₂ and Fc antibody fragments immobilized on functionalized graphene surfaces. Score plot of PC 1 vs. PC 2 for the developed PCA model for PBASE- and TCPP-functionalized graphene surfaces. A TOF-SIMS peak list of 36 amino acid mass fragments was utilized. The ellipses drawn around each of the groups represent the 95% confidence limit for that group on PC1 and PC2.

The loading plot for PC2 reveals compositional differences in amino acid of the outermost layer of the immobilized F(ab')₂ and Fc fragments, as shown in Figure 4-14.

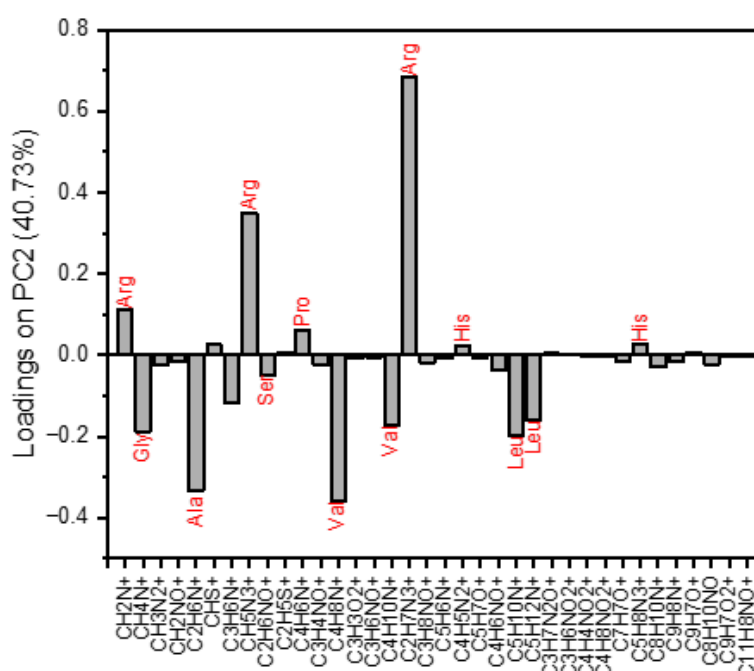


Figure 4-14 Loading plot for PC2 in the developed PCA model for the recorded TOF-SIMS. (Figure 4-13) Ion fragments of amino acids abundant in the Fc domain load in the positive direction, while fragments of amino acids with higher content in the F(ab')₂ domain load in the negative direction. The loadings are ordered in increasing mass-to-charge ratio.

The analysis of loadings on PC2 from the PCA model provide insight into the contributions of amino acid of the protein composition to the separation of F(ab')₂ and Fc fragments. The molecular structures of the F(ab')₂ and Fc fragments of IgG are available from the protein data bank. Therefore, their corresponding amino acid compositions were extracted and used for the calculation of the ratio of F(ab')₂ to Fc fragments in the IgG (Table 4-3). This Fc/F(ab')₂ ratio indicates the prevalence of one amino acid in F(ab')₂ or Fc. A ratio of less than 1 indicated

more prevalence of F(ab')₂ over Fc, which should also be indicated by the negative loadings on PC2. Based on the distinctly negative loadings on PC2, amino acids such as glycine (Gly), valine (Val), Serine (Ser), and alanine (Ala) can be identified as more prevalent for the F(ab')₂ fragment of IgG. This is in good agreement with the results from the TOF-SIMS analysis and the amino acid composition of IgG from the protein data bank. Amino acids such as arginine (Arg), histidine (His) and proline (Pro) are characteristic for the Fc fragment of IgG. The relative concentrations of these amino acids correlate well with the results obtained from TOF-SIMS (Table 4-4). This indicates the high reliability and accuracy of the results of the PCA analysis of the TOF-SIMS data, which can well effectively separate and characterize the graphene surfaces immobilized with F(ab')₂ and Fc based on their slightly different amino acid compositions.

Table 4-3 Amino acid composition of F(ab')₂ and Fc fragments from IgG and their corresponding F(ab')₂/Fc ratios

Amino acid	F(ab') ₂		Fc		Ratio of Fc/F(ab') ₂ composition
	Number of groups	Composition %	Number of groups	Composition %	
alanine (A)	44	5.14	8	3.65	0.71
arginine (R)	20	2.34	11	5.02	2.15
asparagine (N)	26	3.04	8	3.65	1.20
aspartate (D)	30	3.50	10	4.57	1.30
cysteine (C)	28	3.27	5	2.28	0.70
glutamate (E)	28	3.27	15	6.85	2.09
glutamine (Q)	34	3.97	9	4.11	1.03
glycine (G)	76	8.88	10	4.57	0.51
histidine (H)	4	0.47	5	2.28	4.89
isoleucine (I)	26	3.04	10	4.57	1.50
leucine (L)	52	6.07	14	6.39	1.05
lysine (K)	34	3.97	15	6.85	1.72
methionine (M)	4	0.47	4	1.83	3.91
phenylalanine (F)	26	3.04	8	3.65	1.20
proline (P)	56	6.54	22	10.05	1.54
serine (S)	100	11.68	21	9.59	0.82
threonine (T)	128	14.95	15	6.85	0.46
tryptophan (W)	16	1.87	4	1.83	0.98
tyrosine (Y)	36	4.21	7	3.20	0.76
valine (V)	88	10.28	18	8.22	0.80

Table 4-4 Comparison of the prevalence of amino acids in the TOF-SIMS analysis with their composition ratios $Fc/F(ab')_2$ extracted from the protein structure.

Amino acids more prevalent in Fc from PCA of TOF-SIMS spectra	$Fc/F(ab')_2$ composition ratio from protein structure
Arg	2.15
His	4.89
Pro	1.54

Amino acids more prevalent in $F(ab')_2$ from PCA of TOF-SIMS spectra	$Fc/F(ab')_2$ composition ratio from protein structure
Gly	0.51
Ala	0.71
Val	0.8
Ser	0.82
Leu	1.05

Figure 4-13 display the plot of scores for principal component 1 (PC1) against principal component 2 (PC2) derived from the PCA model for the functionalized graphene substrates. It can be seen that the plot of PC1 showed a good separation between the presence and absence of protein on substrates, while the PC2 plot can clearly distinguish between the various proteins groups immobilized on the graphene surfaces. Specifically, substrates with immobilized $F(ab')_2$ fragments exhibited negative loading in PC2, while those with immobilized Fc fragments showed positive loading (Figure 4-13).

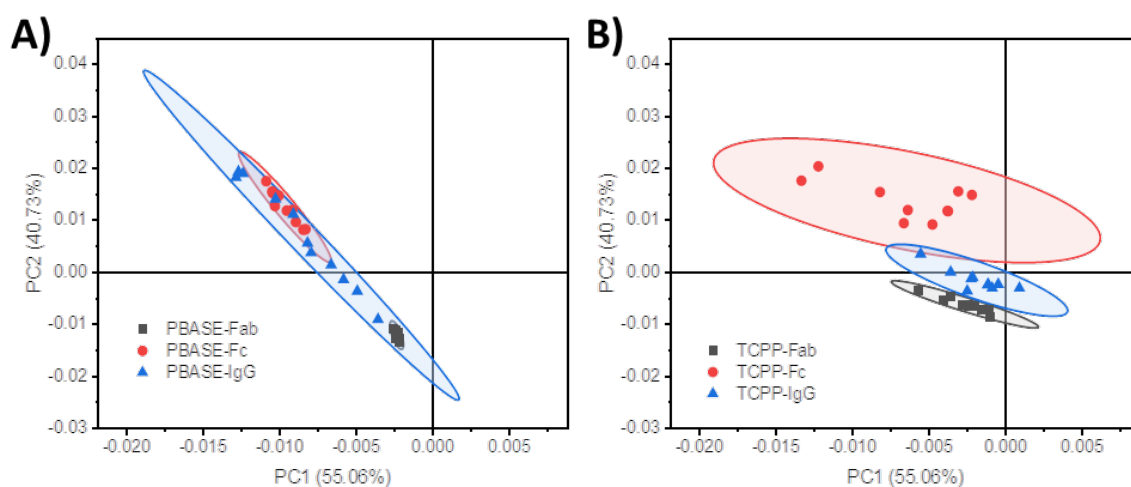


Figure 4-15 Principal component analysis of the antibody fragments ($F(ab')_2$ and Fc) and IgG immobilized on functionalized graphene surfaces, showing PC 1 vs. PC 2 score plot for the developed PCA model for: (A) PBASE functionalized graphene surfaces; and (B) TCPP functionalized graphene surfaces. A TOF-SIMS peak list of 36 amino acid mass fragments was utilized. The ellipses drawn around each of the groups represent the 95% confidence limit for that group on PC1 and PC2.

The PCA analysis of TOF-SIMS data can clearly separate and characterize the graphene surfaces immobilized with $F(ab')_2$ and Fc based on their slightly different amino acid compositions. The relative concentrations of the characteristic amino acid composition, sourced from the protein data bank, correlates well with the findings from the TOF-SIMS analysis (Figure 4-13 and Figure 4-14 and Table 4-4). This consistency underscores the reliability and precision of the results extracted from the PCA analysis of the TOF-SIMS data.

Further analysis of the PC2 scores offers perspective on how the orientation of surface-immobilized IgG differs between the graphene functionalization linker molecules, TCPP and PBASE. In particular, the orientations of the IgG adsorbed on graphene leads to differences in the exposure of the $F(ab')_2$ and Fc fragments on the surface. From Figure 3B, it can be concluded that the outer regions of IgG adsorbed on PBASE-functionalized graphene surfaces correspond to both Fc and $F(ab')_2$ fragments, indicating mixed head-on, end-on and side-on orientations of the IgG antibodies. In contrast, the outer regions of IgG adsorbed on TCPP-functionalized graphene are dominated by the $F(ab')_2$ fragments of the molecule (Figure 4-15), indicating a dominant end-on vertical orientation. This observation aligns well with the conclusions drawn from the electrical sensing measurements for the developed GFET sensors and the microscopy characterization results. More accessible binding sites on the antibody molecule are expected when the antibody is adsorbed onto TCPP-functionalized graphene, since an end-on orientation of antibodies on graphene ($F(ab')_2$ up) is predicted. Therefore, a higher target analyte capture rate and binding efficiency is expected. Hence the TCPP-functionalized GFET sensors are predicted to exhibit increased device sensitivity.

4.5 DFT simulation

In an attempt to explain the experimental results and the effect of the linker molecular structure on antibody orientation, DFT simulations were performed so as to determine the configuration of PBASE and TCPP on graphene for the prediction of their corresponding conjugated antibody orientations.

The structure of the PBASE molecules was first optimized in vacuum prior to their adsorption onto graphene. The most stable configuration of PBASE molecules, denoted as PBASE0 and shown in Figure 4-16, has a flat alkyl chain. This configuration is the most energetically favourable configuration, as corroborated by previously published literature.[267]

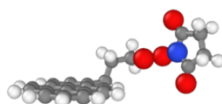


Figure 4-16 Configuration of PBASE0, with atoms coloured as follows: grey = C; white = H; red = O; blue = N.

PBASE0 is used as the initial configuration of the PBASE molecule to construct a set of PBASE configurations by introducing different rotations of the alkyl chain. This rotation was achieved by manipulating various $\text{CH}_2\text{-CH}_2$ bonds or the C-O-N bond in the succinimide ester backbone of PBASE (Figure 4-17). All the configurations were optimized with different rotation angles to gauge their relative stability and the energy profiles calculated for specific PBASE configurations at each rotation are presented in Figure 4-17 and Figure 4-18. These mixed configurations demonstrate the high flexibility of the alkyl chain in the PBASE molecule, suggesting that the succinimide ester backbone can adapt in multiple orientation, leading to diverse antibody orientations on graphene biosensors.

Subsequently, the configurations of PBASE molecules on the surface of graphene were identified and calculated the corresponding adsorption energy, E_{ads} , using the formula:

$$E_{ads} = E_{PBASE/graphene} - E_{graphene} - E_{PBASE}$$

where $E_{PBASE/graphene}$ is the total energy of PBASE adsorbed on the graphene, $E_{graphene}$ is the energy of the graphene, and E_{PBASE} is the energy of PBASE. All results were converged in the same size box.

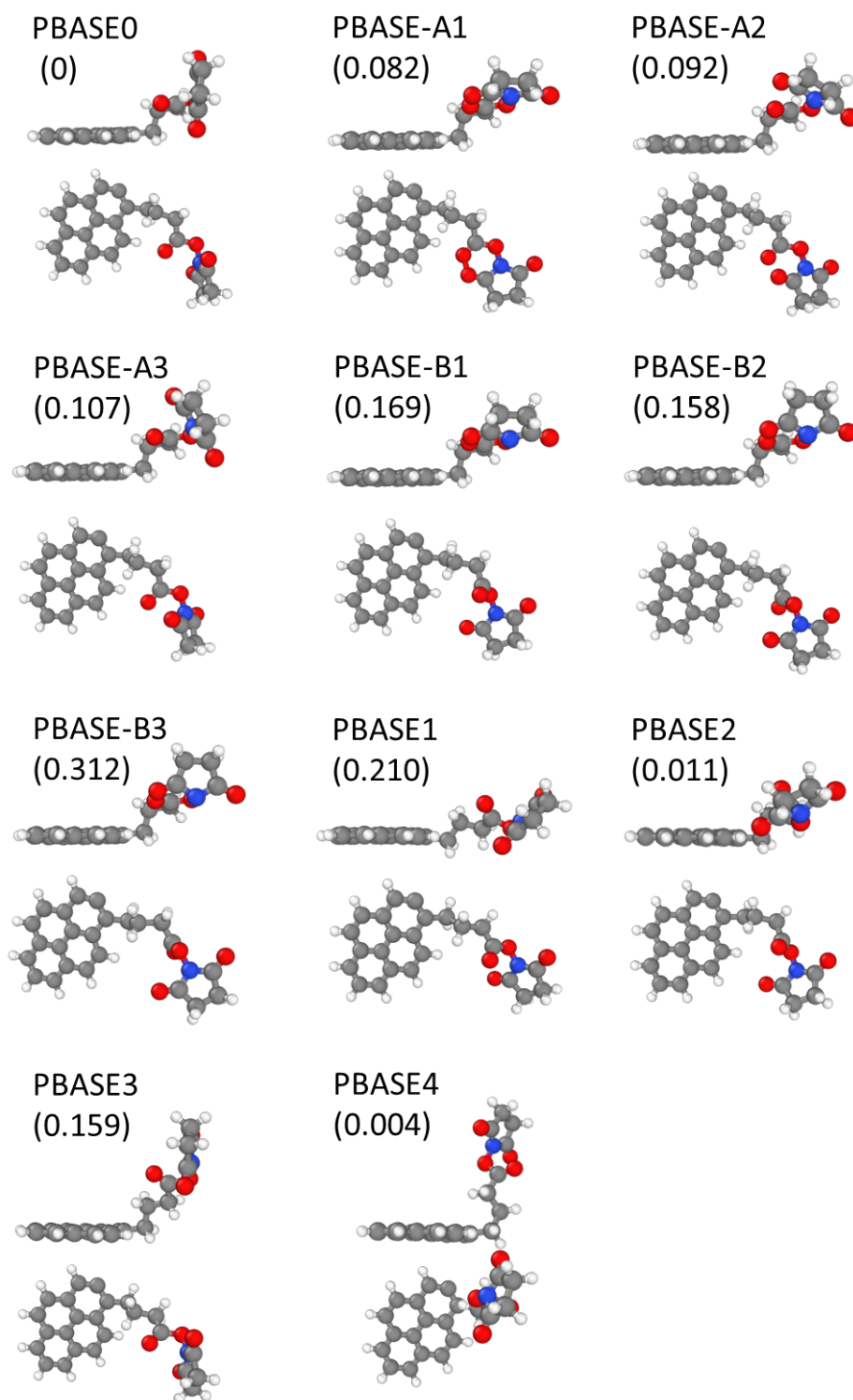


Figure 4-17 Configurations of PBASE with different rotations of its alkyl chain. The corresponding energy of each specific configuration of PBASE is shown in bracket. Atoms are coloured as follows: grey = C; white = H; red = O; blue = N.

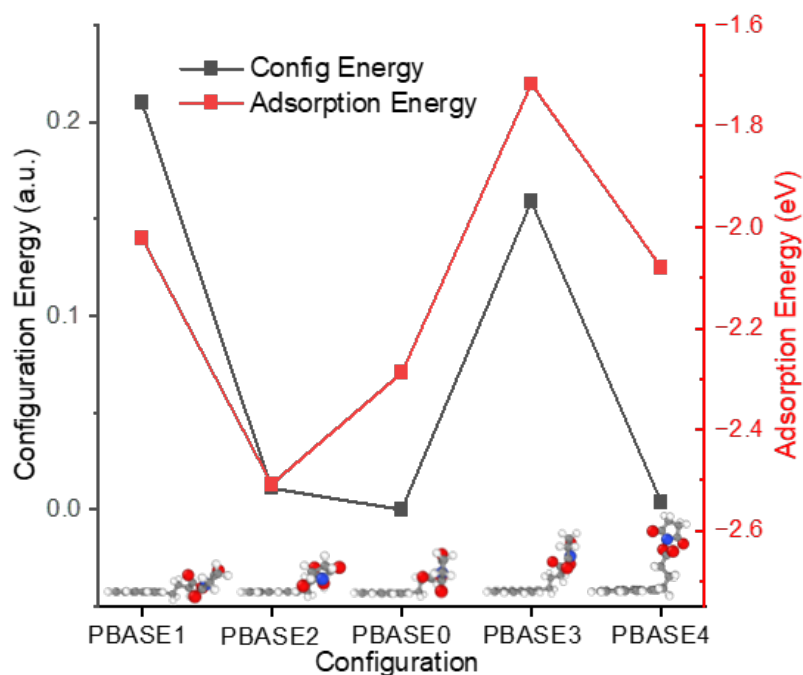


Figure 4-18 DFT simulation of optimized PBASE structures on the graphene substrate for configurations with different angles of rotation. The PBASE0 configuration is used as the initial configuration before the rotation of the bonds. The relative energy of the PBASE molecule is a function of its configuration with different degrees of the angle of rotation. Black lines correspond to the configuration energy of the PBASE molecule as a function of the rotation angle, and red lines correspond to the adsorption energy of the PBASE configuration with respect to binding on the graphene surface.

Using the configurations of PBASE molecules with different rotations as representative models of PBASE molecules adsorbed on the graphene surface (Figure 4-18 and Figure 4-19), the selected geometry combinations were optimized using the first principal based DFT theory.

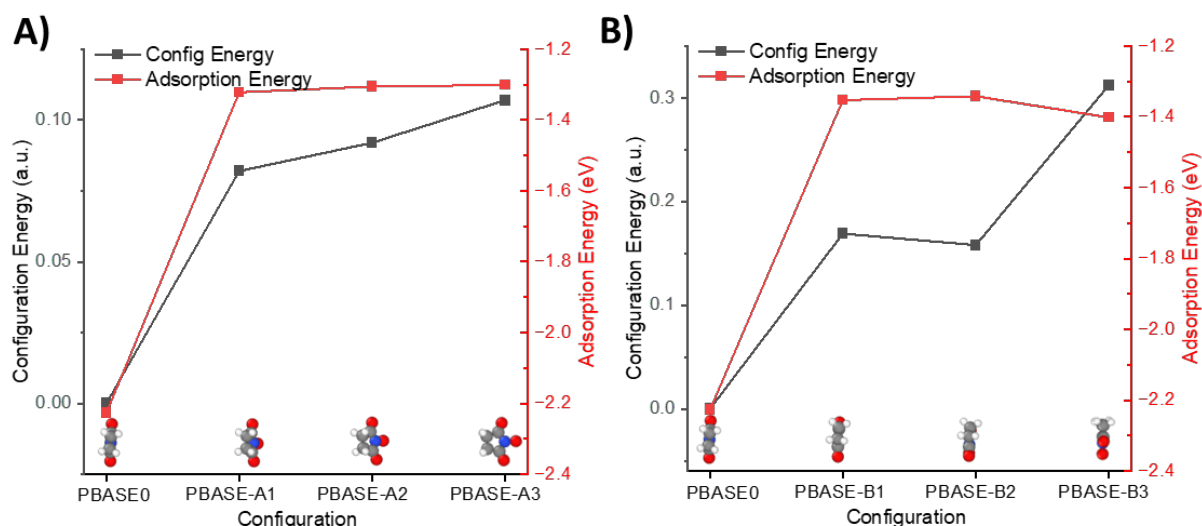


Figure 4-19 DFT simulation of optimized PBASE structures on the graphene substrate for configurations with different angles of rotation. The PBASE0 configuration is used as the initial configuration before rotation of the bonds, marked as the 0 angle. The relative energy of the PBASE molecule is a function of its configuration with different rotation angles. Black lines refer to the configuration energy of the PBASE molecule as a function of the rotation angle, red lines correspond to the adsorption energy of the PBASE configurations with respect to binding on the graphene surface. (A) Rotation 1 around C-O-N (PBASE 0, PBASE-A1-3 in Figure S6); (B) Rotation 2 around C-O-N (PBASE 0, PBASE-B1-3 in Figure S6). The rotation of the PBASE end group is demonstrated at the bottom of each plot. Atoms are coloured as follows: grey = C; white = H; red = O; blue = N.

As summarized in Table 4-5, the adsorption energies for all tested PBASE configurations are negative, signifying exothermic adsorption behaviour. A larger negative E_{ads} value implies a more stable configuration.

Table 4-5 Adsorption energy of each PBASE configuration adsorbed on the graphene surface.

Adsorbed structure on graphene	Adsorption energy (eV)
PBASE0	-2.23
PBASE – A1	-1.32
PBASE – A2	-1.31
PBASE – A3	-1.30

PBASE – B1	–1.35
PBASE – B2	–1.34
PBASE – B3	–1.40
PBASE1	–2.02
PBASE 2	–2.51
PBASE 3	–1.72
PBASE4	–2.08

Among them, the PBASE2 configuration on graphene has the lowest adsorption energy, indicating its greater stability compared to other PBASE configurations. These findings indicate that, while the pyrene fragment of PBASE is adsorbed on graphene, the flat PBASE2 configuration of the succinimidyl ester backbone of PBASE (Figure 4-20), is more prevalent and will remain flat after the adsorption. Consequently, the orientation of the antibody on graphene is predicted to be flat. The results obtained from DFT, concerning the PBASE molecule configuration on graphene, resonate with the findings from the PCA analysis of TOF-SIMS data and the electrical measurements.

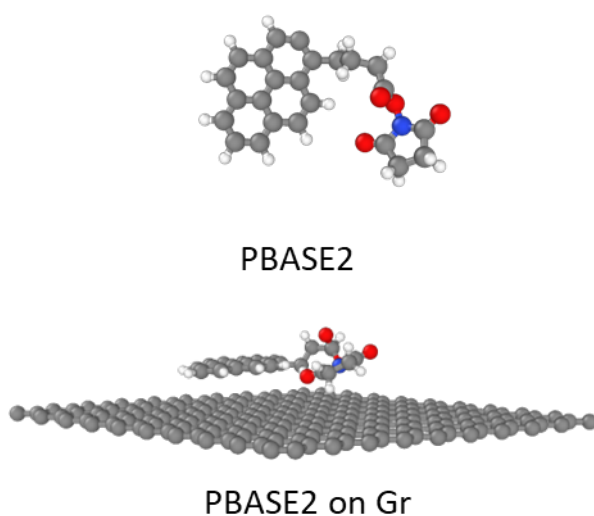


Figure 4-20 DFT simulation of configurations of PBASE. Optimized structure of PBASE2 and its configuration upon adsorption onto graphene (grey: C; white: H; red: O; blue: N).

Additionally, the DFT calculations for the charge density map when PBASE is adsorbed onto the graphene surface (Figure 4-21) indicate a partial charge transfer between the PBASE molecules and the graphene substrate.

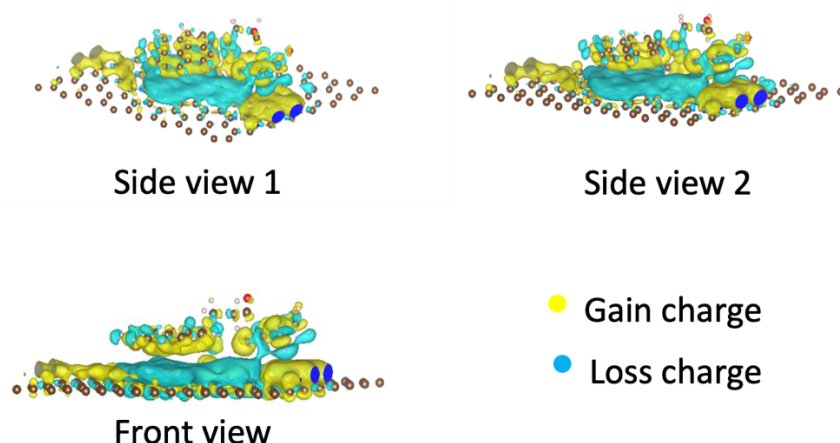


Figure 4-21 DFT calculation of charge density difference map when PBASE2 is adsorbed onto graphene in different views. In the charge density of isosurfaces, yellow indicates regions gaining charge and blue indicates regions losing charge. Cyan and yellow indicate charge accumulation and depletion respectively.

The adsorbed PBASE molecules appear to donate holes to graphene, resulting in p-doping, which is in agreement with the electrical measurement results (Figure 4-22).

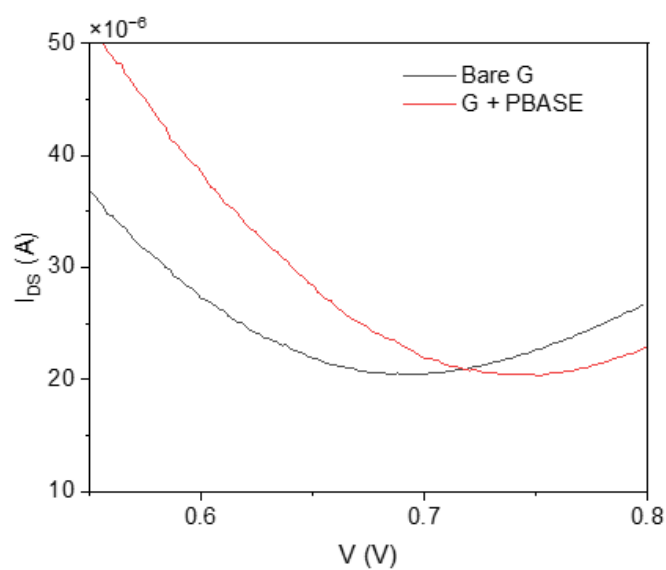


Figure 4-22 Characteristic I–V curves of GFET showing changes in the Dirac point after surface functionalization with PBASE.

Compared to PBASE, the TCPP molecule features four phenol rings that are attached to the carbon atoms of the central porphyrin ring. The structure of the TCPP molecule was fully relaxed using the DFT method as illustrated in Figure 4-23 and Figure 4-24.

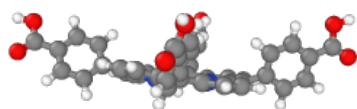


Figure 4-23 Optimized geometry of TCPP molecule. Atoms are coloured as follows: grey = C; white = H; red: O; blue = N.

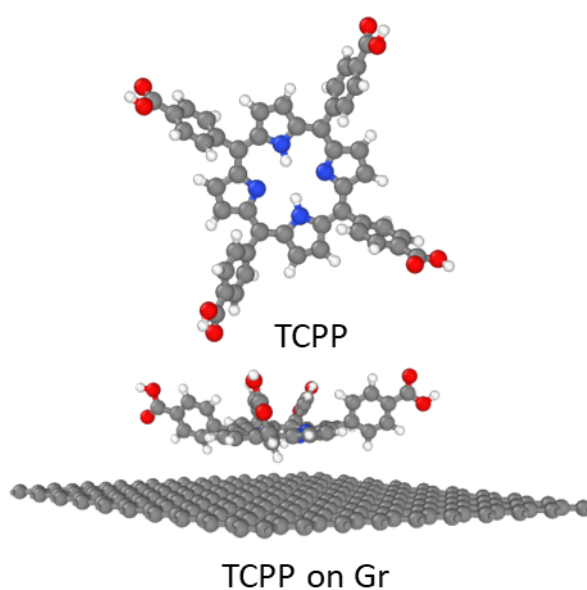


Figure 4-24 Optimized structure of TCPP, and its configuration upon adsorption onto graphene (grey: C; white: H; red: O; blue: N).

The DFT simulations show that these phenol rings are not located in the same plane as the central porphyrin ring. When the porphyrin molecule is adsorbed onto graphene, the aromatic porphyrin rings remain flat on the graphene surface.[268], [269], [270], [271], [272] The peripheral rings arranged around the central porphyrin ring appear at a rotated angle which is bent up and tilted away from the porphyrin plane due to steric hinderance.[268], [269] The peripheral rings are thus twisted for maximizing the contact between the aromatic porphyrin rings and graphene. (Figure 4-23 and Figure 4-24) These findings indicate that the carboxylic groups of the TCPP molecule are bent up and tilted away from the graphene surface that enable the amine groups on antibody to bind after activation. As a result, upon conjugation with antibodies, the antibody orientation on graphene is predicted to be upright. The conclusion drawn from the DFT study, corroborated by literature[268], [269], [273] confirm the findings from the PCA analysis of TOF-SIMS data and the electrical measurements.

4.6 Summary

A biofunctionalization technique for graphene using the TCPP linker is developed, which enables the appropriate orientation of antibodies on the graphene surface. To elucidate the heightened GFET sensitivity, TOF-SIMS was used to investigate the antibody orientation on graphene surfaces functionalized with both TCPP and with PBASE. For this study, an IgG antibody with a known amino acid sequence is selected. The TOF-SIMS analysis revealed that the IgG adsorbed on TCPP-functionalized graphene has predominantly an end-on vertical orientation. In contrast, IgG antibodies adsorbed on PBASE-functionalized graphene exhibited a mix of head-on, end-on and side-on orientations. DFT simulations indicated that the succinimidyl ester backbone can adapt various directions, resulting in diverse antibody orientations on graphene biosensors, due to the pronounced flexibility of the alkyl chain in the PBASE molecule. The simulation further suggested that the flat configuration of the succinimidyl ester backbone in PBASE is more energetically favorable, predicting a flat orientation of antibody on graphene.

With the newly developed TCPP method, it is demonstrated that this approach can refine the orientation of conjugated antibodies on graphene surfaces, thereby it may markedly boosting

the performance of GFET biosensors. Therefore, the next chapter will investigate the influence of linker molecule of the performance of GFETs biosensors.

5 Role of linker molecules in performance of graphene biosensors

5.1 Key Novelty and Achievements

After the proof of concept of the developed TCPP method in enhancing the antibody orientation on the graphene surface. This Chapter aims to evaluate the performance of graphene field-effect-transistor based biosensors for cancer exosomes detection in buffer and real clinical samples.

The new functionalization technique is applied on GFET biosensors to detect pancreatic cancer exosomes in patient-derived plasma samples, highlighting its potential to redefine early disease diagnosis. By adopting the TCPP-functionalization method, a two orders of magnitude improvement in the Limit of Detection (LoD) of GFET biosensors for exosome biomarkers, compared to the commonly PBASE-functionalized GFETs is achieved. Additionally, the TCPP-functionalized graphene surface displayed a threefold surge in exosome density.

In this chapter, in order to facilitate the biosensing with liquid samples, a house designed and fabricated microfluidic reservoir was implemented. Details related to the design and fabrication steps are introduced in Chapter 3.6.4. To avoid the need of bulky reference electrodes and to develop an on-chip integrated GFET platform for the point-of-care testing, an on-chip integrated Au electrode is designed and tested. In order to ensure the device uniformity and minimize the false-positive signals, a differential measurement with separated control and sensing channel is designed and implemented.

The work presented in this Chapter is part of the Journal publication: Tianyi et al. Control of antibody orientation on graphene using porphyrin linker molecules for high-performance graphene-based immuno-biosensors,” Submitted.

The portable electronic device was designed by Dr. Bruno Gil Rosa. Clinical samples were tested using foundry fabricated chips from Graphenea.

5.2 Overview

After the proof-of-concept for the investigation of antibody orientation on the surface of graphene, which is described in the previous chapter. In this work, the hypothesis of improving the performance of GFET biosensors functionalized with tetrakis (4-carboxyphenyl) porphyrin (TCPP) rather than the widely used PBASE molecule was studied. This technique was further applied to detect pancreatic cancer exosomes in patient-derived plasma samples, highlighting its potential to redefine early disease diagnosis. The performance of graphene field-effect-transistor based biosensors for cancer exosomes detection in buffer and real clinical samples were evaluated for both PBASE- and TCPP- functionalized GFET biosensor.

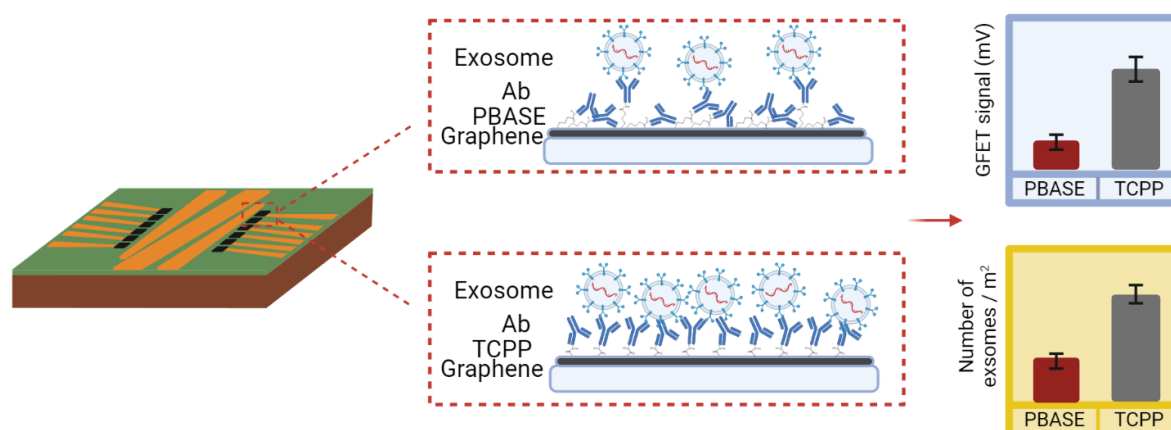


Figure 5-1 Schematic illustration of the PBASE-functionalized GFET and TCPP-functionalized GFET sensors. Schematic illustrations of the exosomes captured on PBASE-functionalized GFET and TCPP-functionalized graphene. The functionalization with TCPP enhances capture of the exosome and leading to higher device sensing signal.

5.3 Exosome and their role in cancer detection

Early-stage cancer screening based on blood tests could allow for large number of populations to be screened for early detection, improve the treatment outcome, and potentially reduce the mortality rate. However, they often suffer from highly inaccurate predictions when the cancer is in the early stage due to the small amount of cancer biomarkers in the blood, leading to unnecessary tissue biopsies and CT/MRI screening. Moreover, using such methods to

screen the general population for cancer is not practical and not cost-effective due to its low incidence.

Recently, the application of liquid biopsy involving biomarkers including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), microRNAs (miRNAs), and exosomes in blood, urine, and saliva for the cancer diagnosis are drawing more and more attention. These novel biomarkers presented in body fluids have emerged as promising approaches for early cancer diagnostics. Among all these biomarkers, exosomes are small extracellular vesicles (50-150 nm) or “bubbles” and are released in high quantities by healthy and tumour cells, enabling cell-to-cell communication and cargo transfer. There is increasing evidence that exosomes released from cancer cells contain disease-specific information that can be used as biomarkers for diagnosis at an early stage or even predict its occurrence years before its outbreak. Furthermore, exosomes are abundant in a patient’s blood sample and there is potentially more than one specific exosome biomarker for each type of cancer. Therefore, the detection of multiple cancer-derived exosomes will potentially allow more accurate cancer detection and at a much earlier stage.

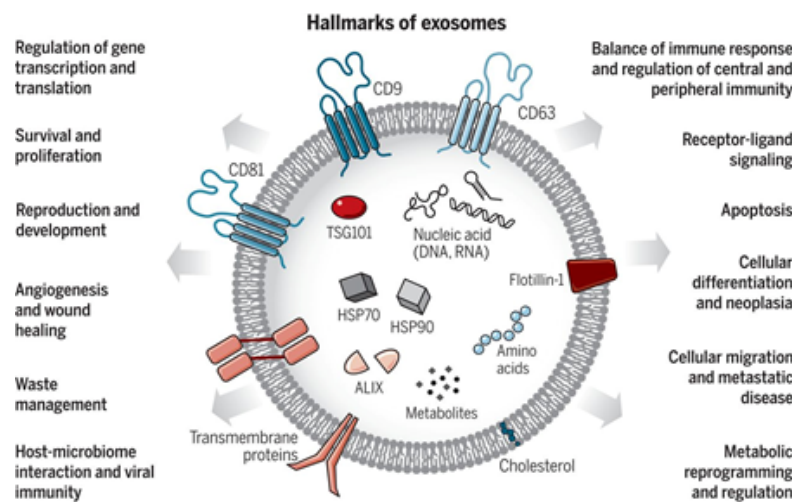


Figure 5-2 Schematic diagram of the structure of an exosome, which is a cell-to-cell transit system with pleiotropic functions, it is consisted of phospholipid bilayer, multiple transmembrane proteins and other contents. [274]

Exosomes carry different biomolecules as cargo, including proteins, lipids, DNA and RNA. Exosomes act as intercellular communicators, with their biomolecular cargo inducing changes in recipient cells.[275] The composition of exosomes is complexed and is highly dependent on the status of its cell type of origin. Generally, exosomes are enriched in plasma membrane lipids, such as cholesterol, phosphatidylserine, sphingomyelin (SM), saturated fatty acids, and hexosylceramides. Exosomes also contain high level of proteins with different functions. These proteins include tetraspanins (typically CD9, CD63, CD81 and CD82), which are involved in exosome formation, cell penetration, invasion, and fusion events; heat shock proteins (HSP70, HSP90), which take part in the cellular response and stress response during antigen binding and presentation; multivesicular body formation proteins (Alix, TSG101) that are involved in the exosome release and formation. Although the composition of exosomes are complexed and heterogeneous, these structural proteins are the characteristic biomarkers for exosomes. (e.g. CD9, CD63, CD81 and TSG101, Alix) Epithelial cell adhesion molecules (EpCAM), and members of the human epidermal growth factor receptor (HER) family are also enriched on exosomes and considered as markers of exosomes. [276], [277], [278]MicroRNA (miRNA) carried within the exosome play a key role in intercellular communicators, as they are a class of short single-stranded non-coding RNA which functions as part of the RNA induced silencing complex to regulate gene expression. MicroRNA (miRNA) carried within the exosome may play a key role in this, as they are a class of short single-stranded non-coding RNA which functions as part of the RNA induced silencing complex to regulate gene expression. Some of these biomolecular cargos are universally packaged into one type of exosome, while others are selectively sorted into exosomes[279]. This makes them potentially useful as biomarkers, giving an insight into the functional state of their originating cell. Exosomes may play a role in many different cancers, where different miRNAs can act as tumour suppressors or tumour promoters when transferred to recipient cells. Exosomes can also modulate changes in the tumour microenvironment to promote tumour growth, metastasis and evasion from the immune system.[280] They may therefore present potential cancer-specific therapeutic targets, and can also be engineered to act as chemotherapy drug carriers. [281] Exosomes are released into the extracellular milieu, therefore available in biofluids such as blood, urine and saliva. Many aspects of exosomes have been researched as potential biomarkers for PC, including exosome concentration, [282] cell surface proteins

including Glypican-1 (GPC-1) [283], and miRNAs [284]. However, there is still more research needed into biofluid exosomes before they are ready to enter clinical use.

5.4 Optimisation of GFETs for the detection of exosomes

5.4.1 GFET sensor array

The GFET sensor array is fabricated first using the transferred graphene on SiO₂/Si substrate. Detailed transfer process and device fabrication process are described in Chapter 3.1 and Chapter 3.3. To investigate the quality of graphene on the fabricated GFETs before performing electrical measurements, optical microscopy was performed first on the fabricated GFETs. Optical microscopy was first performed to examine the quality of graphene on the GFET after the device patterning and metallisation, as shown in Figure 5-3 A. Device passivation was performed with a 1.7 μ m SU8 film on the metal contacts, which can reduce the parasitic effects and passivate the device during liquid measurements. Figure 5-3 B shows the quality of the graphene after the SU8 passivation. The quality of the graphene before and after SU8 passivation are both good without any defects shown. The fabricated GFET device shows a continuous monolayer of graphene with no cracks, impurities or wrinkles shown. The house fabricated GFET sensory array was found to have high reproducibility with high graphene and device quality.

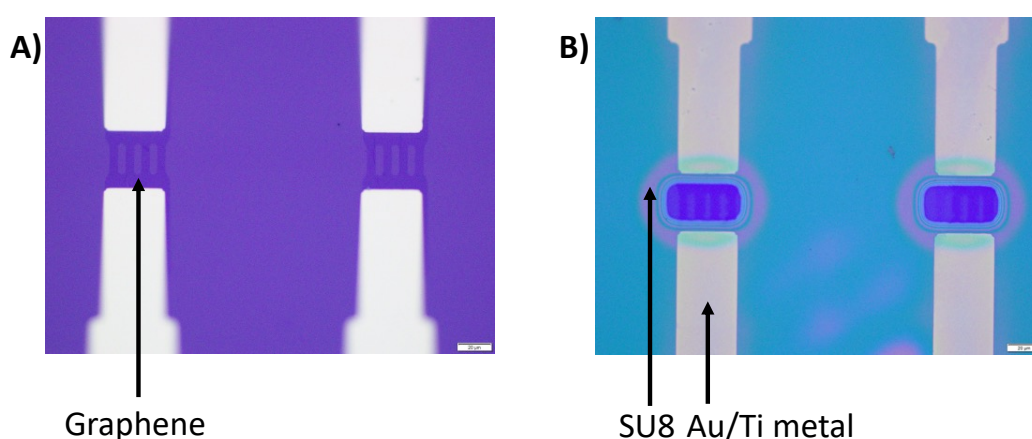


Figure 5-3 Optical microscopy images of the GFET device A) before the passivation B) after the passivation. Images are taken at 20x magnification.

5.4.2 On-chip gold gate electrode

The fabricated GFETs sensor array was then used for electrical measurements with the buffer solution. Three different GFET configurations were tested and compared, with Ag/AgCl electrode, with Pt electrode, and with the on-chip Au electrode.

Electrical measurements were first performed on the fabricated GFET with all three different electrodes, Ag/AgCl electrode, Pt electrode, and the on-chip integrated Au electrode, in PBS buffer solution. The characteristic I-V curves of the tested GFETs are shown in Figure 5-4. It is shown that all the fabricated GFET sensor showed a response upon the introduction of PBS buffer solution. There are 30 devices on each in-house fabricated GFET chip, all of the house fabricated GFET sensors tested showed consistent responses with high reproducibility. It is shown that the Dirac points for the GFET biosensor with Pt electrode, Ag/AgCl electrode, and on-chip integrated Au electrode are different. This change is due to the difference in work function between Pt and Au with the buffer solution and graphene.

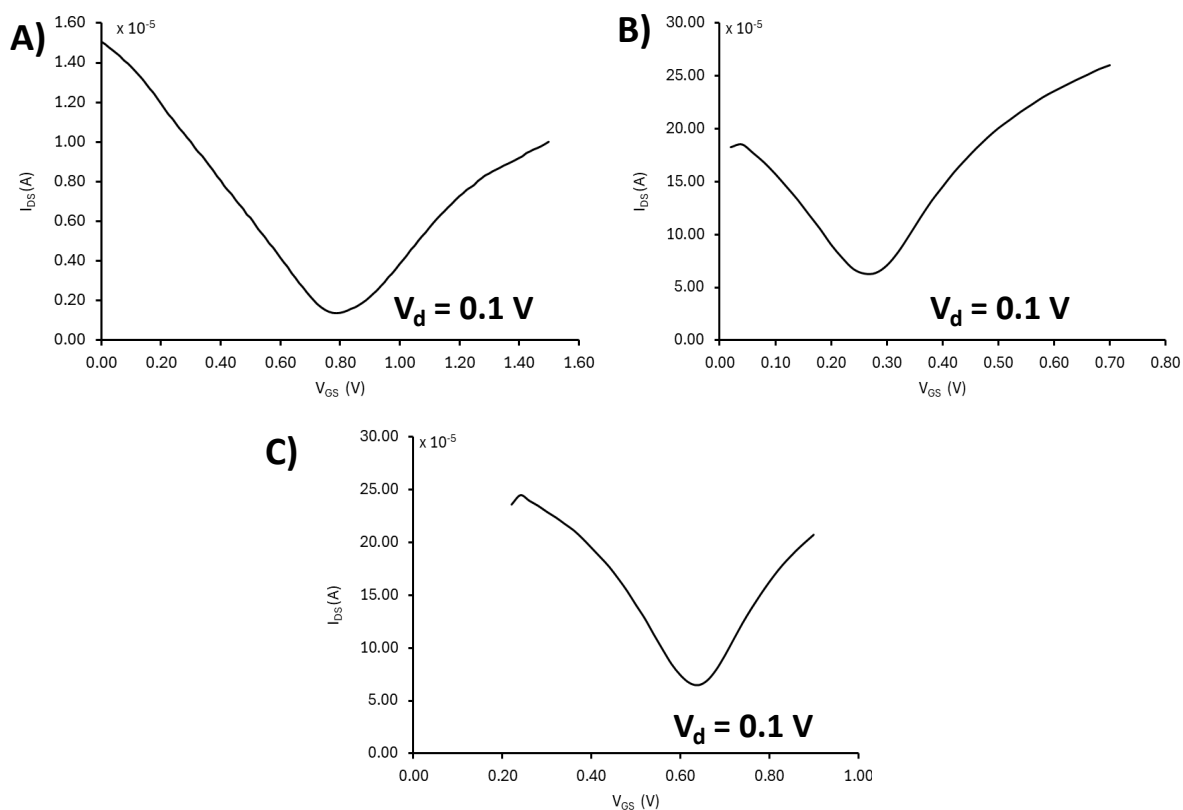


Figure 5-4 Validation of the performance of fabricated GFET biosensor with solution gate. Representative I_{DS} - V_{GS} curve of the GFET biosensor with A) Pt wire gate electrode, the tested GFET devices are with a graphene channel area of $20\ \mu\text{m} \times 10\ \mu\text{m}$, B) Ag/AgCl gate electrode, and C) on-chip integrated Au electrode. The response recorded with the Ag/AgCl gate electrode (B) and on-chip integrated Au electrode (C) are tested using the same GFET device with a graphene channel area of $100\ \mu\text{m} \times 100\ \mu\text{m}$. The drain voltage V_d for the electrical measurements are labelled in the plot. All measurements are performed using $0.001\ \text{x PBS}$.

For the fabricated GFET sensor with Pt wire as the gate electrode, it is found that different fabricated showed same good response with a consistent characteristic I-V curve shown. (Figure 5-5)

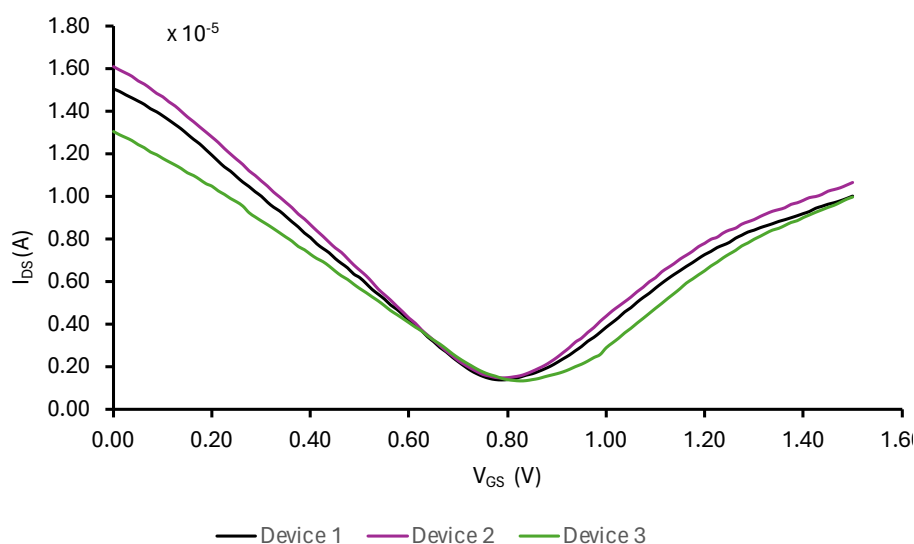


Figure 5-5 Representative I_{DS} - V_{GS} curve of the GFET biosensor with the Pt wire gate electrode at 3 different devices. The drain voltage V_d for the electrical measurements are set as 0.1 V. All measurements are performed using 0.001 x PBS. The fabricated GFET devices are with a graphene channel area of $20\ \mu\text{m} \times 10\ \mu\text{m}$.

In comparison to the conventional GFET single sensor which needs a bulky external gate electrode (such as Ag/AgCl), an on-chip GFET sensor is advantageous not only because of the use of an integrated on-chip gate/reference electrode in the centre area which avoids external bulk electrodes and a complicated set-up.

To further quantify stability of on-chip integrated Au electrode, electrical measurements were performed. The stability of the devices in PBS were tested by recording the changes in transfer curves after repeating the measurements several times. It is observed that devices showed some drift, which can range from a few mV for some chips up to 200 mV in others. However, the drift usually vanishes after the first five measurements and the transfer curve becomes stable (Figure 5-6). The origin of this drift behaviour could originate from the charge imbalance attributed to gold dissolution, as has been reported for aqueous solutions.[285] Dissolution has been linked to defects present on the gold surface; namely, in the different ionic states of Au, O/OH adsorption and Au–O/OH place exchange. This process could take a few cycles to saturate in the case of an unpolished/uncycled sample due to the saturation of the surface towards a reversible regime. [285] Therefore, during the measurements of sensing each measurement was repeated five times before recording the transfer curve. The

measurement stability in PBS is further determined by recording the change in transfer curves over time. The I - V curve shows a stable Dirac voltage from 15 to 120 min (Figure 5-7), indicating good stability of the GFET device in buffer solution.

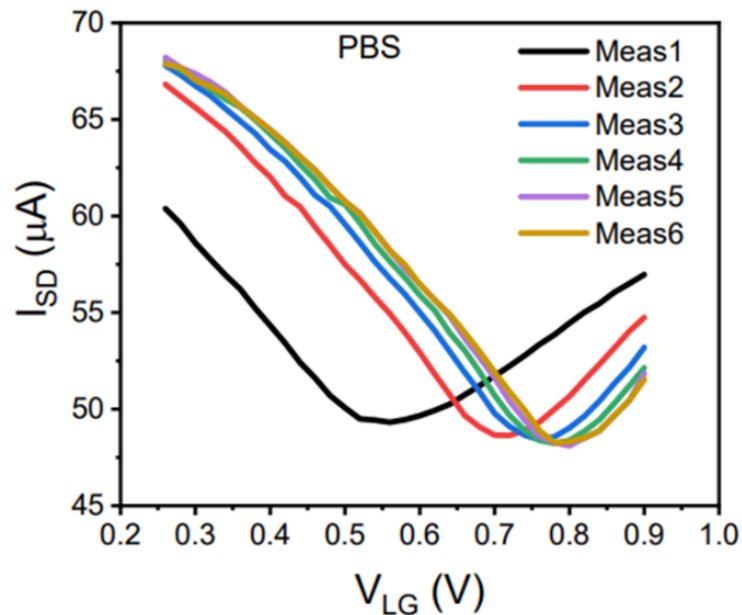


Figure 5-6 Series of repeated transfer curve measurements of GFETs with on-chip integrated Au electrode in PBS. The data shows that the transfer curve become stable after the 5th measurements. The measurements were performed on more than 500 chips and the results were found consistent in more than 500 chips.

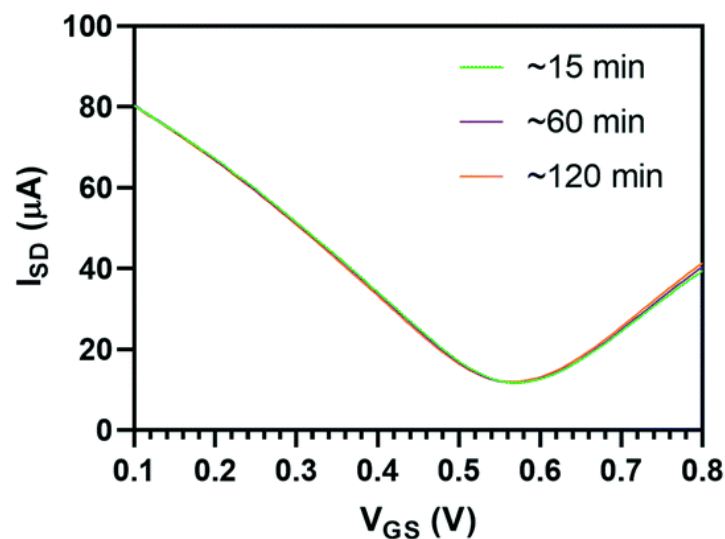


Figure 5-7 Monitoring of I–V curves over time on the same device. $V_{sd} = 20$ mV, indicating good stability of the data produced by each device over time.

5.4.3 Differential measurement

Incorporating internal controls within one detection chip is necessary in order to distinguish between target and interfering molecules or background noise, which enables chip calibration. In this design, the six GFETs in each functional area usually operate as independent internal controls during the same chip measurements and enable the statistical analysis and monitoring of device-to-device variation, facilitating an error-reducing detection process.

To illustrate the role of internal control in improving the accuracy of the detection, 10 ng/ μ L exosomes was spiked in the buffer and tested the GFETs devices response from multiple chips. As shown in Figure 5-8, the introduction of exosomes results in large and inconsistent shifts in both control and sensing channels. However, the difference between the GFET response from control and sensing channels remains the same.

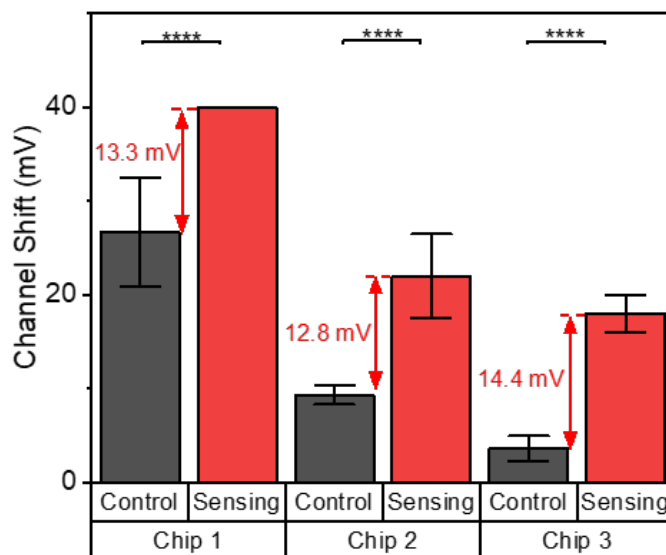


Figure 5-8 GFET device shift of the control and sensing channels on three GFET chips tested for the same concentration of exosomes in buffer, illustrating the importance of control channel. The GFET device detection signal is calculated based on the difference between the GFET response from control and sensing channels. Error bars are determined by the standard deviation of multiple device measurements, $5 \leq n \leq 6$ for each reading.

Furthermore, the GFETs devices response of different chips with various spiked model exosome concentrations from 10^{-2} $\mu\text{g}/\mu\text{L}$ to 1 $\mu\text{g}/\mu\text{L}$ in plasma were measured. MCF-7 exosomes were selected as the model exosomes to demonstrate the analytical performance of the developed GFET sensors, since GPC-1 protein biomarker is also enriched on the surface of MCF-7 exosomes.[286], [287], [288], [289], [290] To confirm the expression of GPC-1 on the surface of MCF-7 exosome, AFM was performed with MCF-7 exosome and GPC-1 antibody. The AFM image is shown in Figure 5-9.

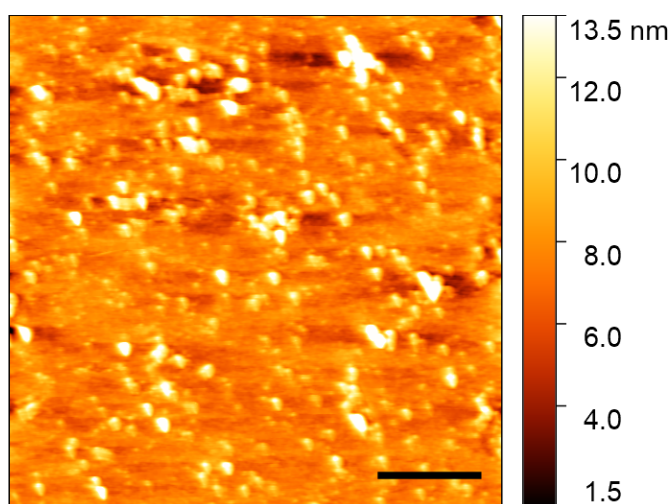


Figure 5-9 Atomic force microscopy images of GPC-1 antibody functionalized graphene surface incubated with MCF-7 exosomes (model exosomes) in buffer, suggesting the expression of GPC-1 on the surface of MCF-7 exosomes. AFM scale bars = 1 μm .

The calibration curve shows strong correlation ($y = 9.92 \log(x) + 44.67$, $R^2 = 0.9990$ and $SD = 0.1700$) between concentration and GFETs response when internal control is used while weaker correlation with higher standard deviation ($y = 17.21 \log(x) + 65.27$, $R^2 = 0.9311$ and $SD = 1.3148$) is observed without the use of control measurements. (Figure 5-10) Graphene

surface is directly interacted with the buffer solution and ions can diffuse and accumulate at graphene surface in addition to multiple interactions at the graphene/buffer interface can result in the drift in the Dirac voltage. As both sensing and control channel undergo the same conditions, therefore the signal difference between sensing and control channels can significantly reduce the error resulted from the drift and increase the reliability of the detection.

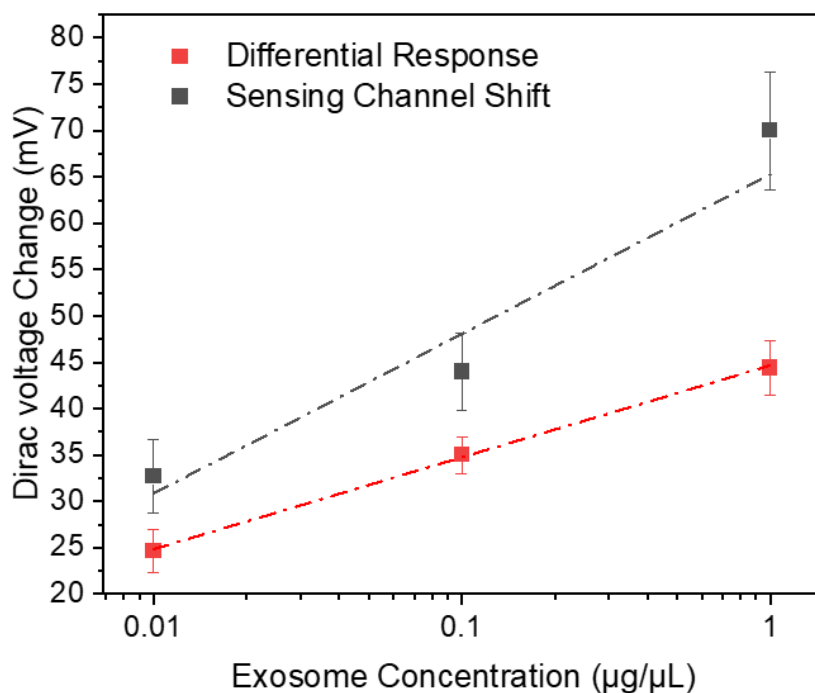


Figure 5-10 Calibration curve of the GFET biosensor for detection of various concentrations of spiked model exosomes in plasma. Error bars are determined by the standard deviation of multiple device measurements, $4 \leq n \leq 24$ for each reading. Differential response represents the device signal, which is the difference between sensing channel and control channel.

5.5 Comparative performance of GFETs with PBASE and TCPP in buffer

5.5.1 Electrical performance

To compare the performance of antibodies conjugated on PBASE- and TCPP-functionalized graphene, the developed functionalized graphene surfaces were fabricated and integrated

into graphene field effect transistors (GFETs). Tests of the biorecognition of exosomes tested with the immobilized antibodies on graphene were performed with the developed GFET biosensors. The functionalized graphene devices with TCPP or PBASE were immobilized with anti-CD63 antibodies and then blocked with bovine serum albumin (BSA) to prevent non-specific binding. Separate control and sensing channels on GFETs were implemented, allowing for testing with the same sample. This was done to compensate for potential device drift and to further reduce the chances of false positive signals from non-specific binding.[20], [230] Measurements of transfer characteristics on the developed GFET sensors were performed to detect exosomes that were spiked into a PBS buffer. The measurements of transfer characteristics for modified GFET sensors indicated that both PBASE and TCPP can well preserve the electronic property of graphene. (Figure 5-11)

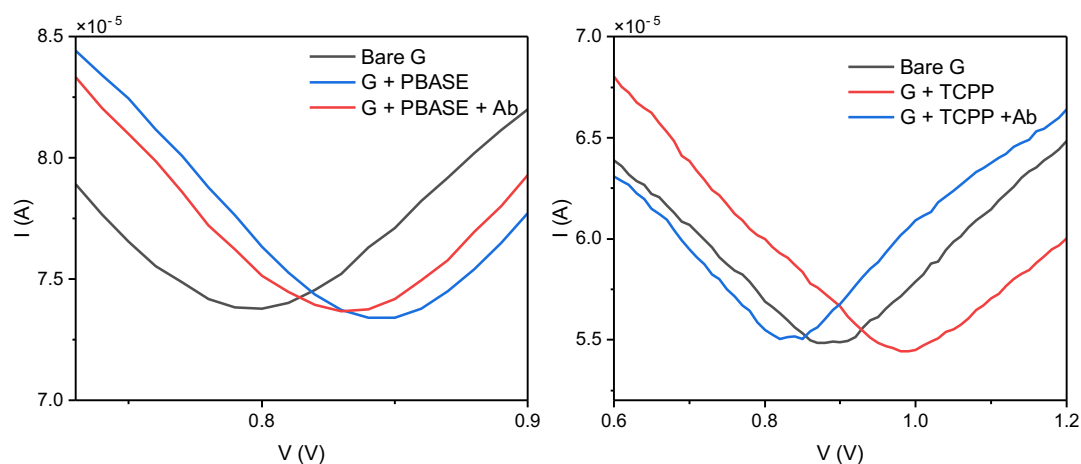


Figure 5-11 Characteristic I–V curves of GFET showing changes in the Dirac point after surface functionalization with PBASE and TCPP.

The I-V curves characteristic of the functionalized GFET biosensors were recorded after 10 min incubation for each concentration with varying concentration of exosomes in PBS buffer, ranging from 10^4 to 10^8 particles/ μ L. Figure 5-12 and Figure 5-13 show a significant leftward shift of the Dirac point with increased exosome concentration for TCPP-functionalized GFET sensors.

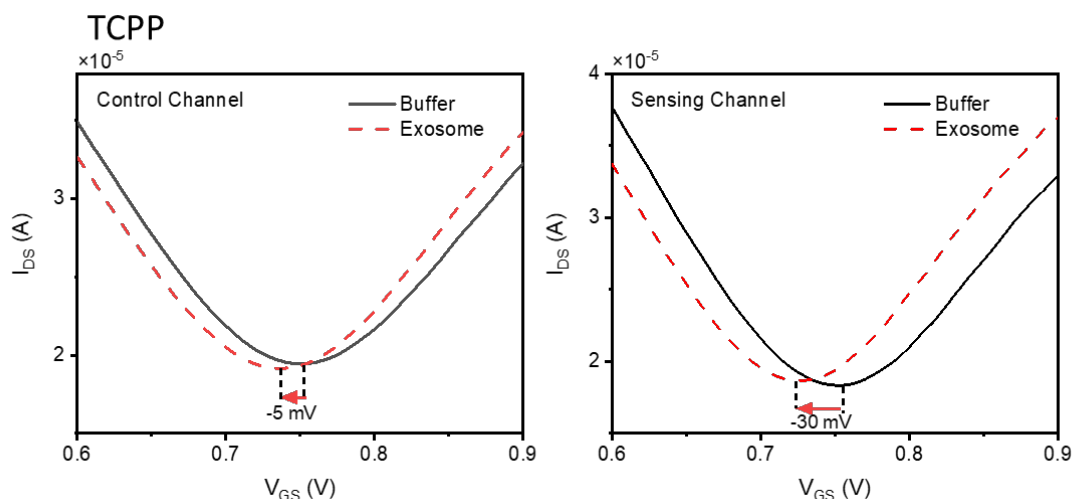


Figure 5-12 Validation of TCPP-functionalized GFET biosensor for detection of exosomes with protocol using control and sensing channels. Detection of cancer exosomes in buffer using the GFET biosensor. Representative I_{DS} - V_{GS} curve of control channel and sensing channel on the GFET biosensor after exosome binding. A significant curve shift is seen in the sensing channel in comparison to the control channel, suggesting that the shift arises from exosome binding on the sensing channel.

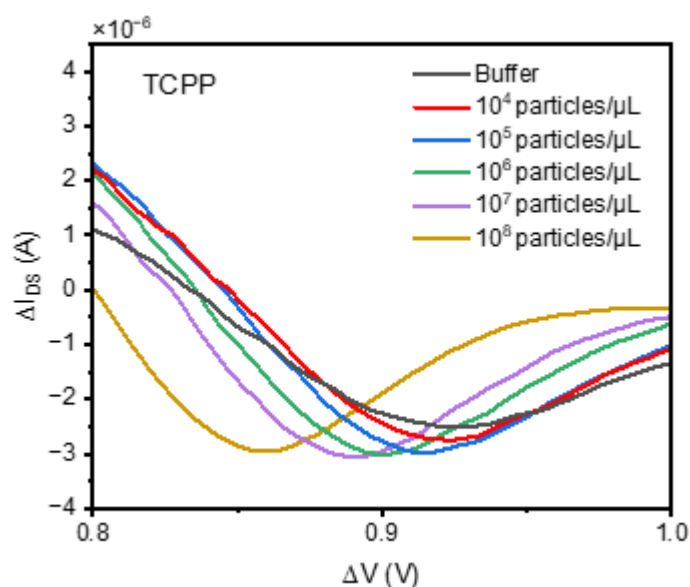


Figure 5-13 Representative differential I_{DS} - V_{GS} curve of the TCPP functionalized GFET biosensors in PBS buffer with various concentrations of spiked exosomes.

In comparison, PBASE-functionalized sensors displayed a minor rightwards shift of the Dirac point upon exosome binding (Figure 5-14 and Figure 5-15).

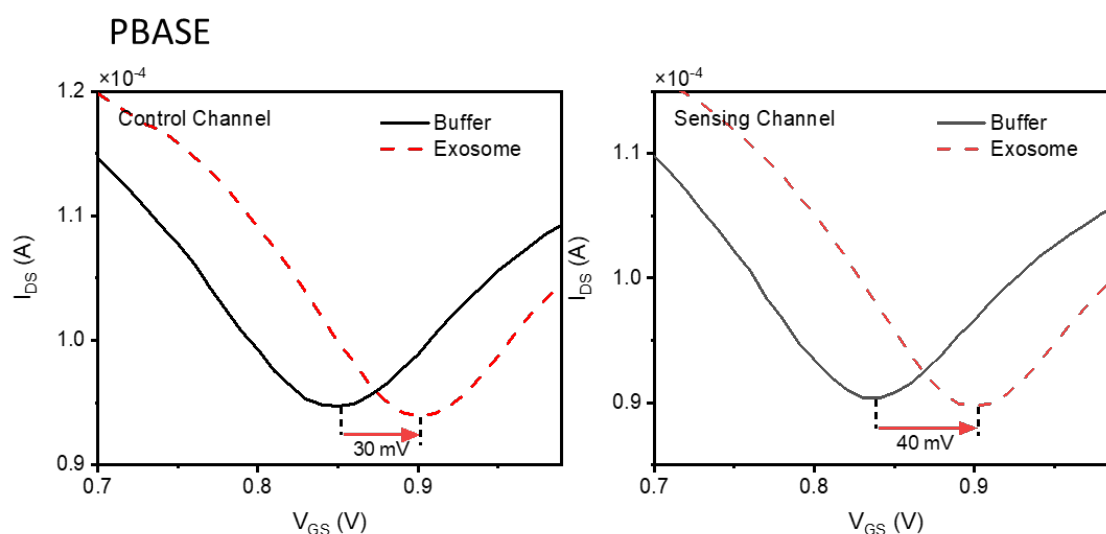


Figure 5-14 Validation of PBASE-functionalized GFET biosensor for detection of exosomes with protocol using control and sensing channels. Detection of cancer exosomes in buffer using the GFET biosensor. Representative I_{DS} - V_{GS} curve of control channel and sensing channel on the GFET biosensor after exosome binding. A significant curve shift is seen in the sensing channel in comparison to the control channel, suggesting that the shift arises from exosome binding on the sensing channel.

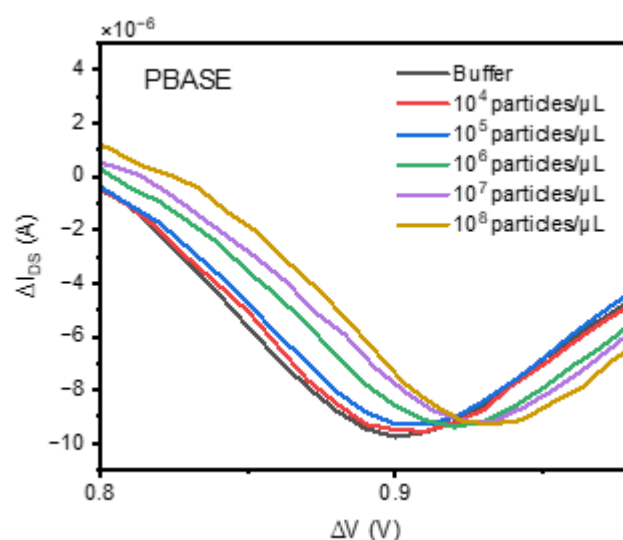


Figure 5-15 Representative differential I_{DS} - V_{GS} curve of the PBASE functionalized GFET biosensors in PBS buffer with various concentrations of spiked exosomes.

In both cases, the Dirac point shifted more with higher exosomes concentration. Figure 5-16 compares of the calibration curves of both TCPP- and PBASE-functionalized GFET biosensors when detecting exosome in PBS buffer. A logarithmic relation was observed between the device shift and spiked exosome concentration for both TCPP- ($y = -10.61 \log(x) + 4.06$, $R^2 = 0.9824$, and $SD = 0.7053$) and PBASE-functionalized GFET sensors ($y = -9.45 \log(x) + 2.52$, $R^2 = 0.9943$, and $SD = 0.5113$). When evaluating the sensing performance, PBASE-functionalized GFET sensors showed a negligible device response for exosomes detection below 10^5 particles/ μL . Conversely, TCPP-functionalized GFET sensors demonstrated a significant device response even at the lower exosome concentration of 10^4 particles/ μL . Interestingly, this response was comparable to that of PBASE-functionalized sensors at a concentration of 10^6 particles/ μL . Furthermore, the limit of detection (LoD) for the TCPP-functionalized sensors was two orders of magnitude higher than that of PBASE-functionalized GFET sensors. The LoD for PBASE-functionalized GFET sensors was calculated using the signal from the pure PBS buffer plus three times the standard deviation of this buffer. Notably, the device response of TCPP functionalized GFET sensors was over twice that of the PBASE sensors, indicating significantly enhanced sensitivity.

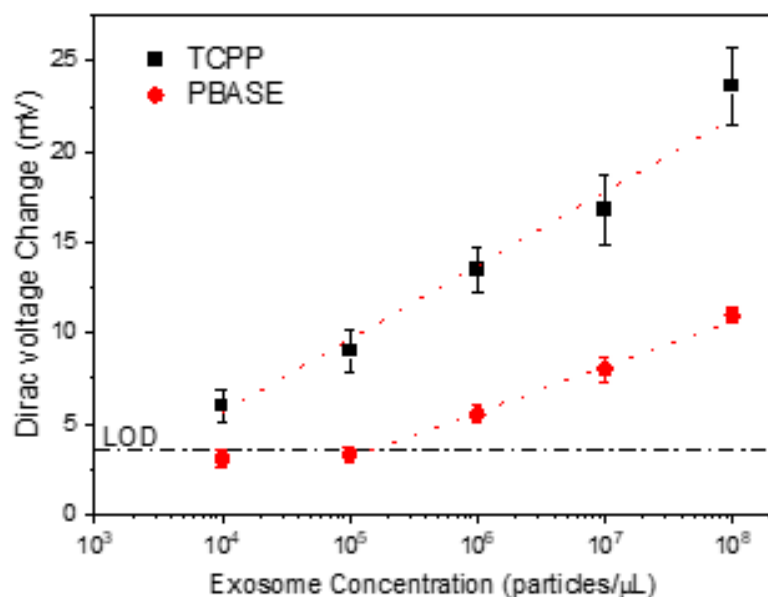


Figure 5-16 Calibration curves of the PBASE and TCPP functionalized biosensor for detection of different concentrations of spiked exosomes in PBS buffer. Error bars are determined by the standard deviation of multiple device measurements, $5 \leq n \leq 24$ for each reading. The baseline that represents LoD is defined by $(\text{response})_{\text{PBS}} + 3 \times \text{standard deviation} = 3.6 \text{ mV}$ ($n = 24$).

5.5.2 AFM results

Subsequently, AFM was conducted to examine the binding of exosomes to surface-immobilized antibodies on PBASE- and TCPP- functionalized graphene surfaces after a 15 minute incubation with a buffer solution of 10^7 particles/ μL concentration (Figure 5-17 and Figure 5-18). Over a total scanned area of $600 \mu\text{m}^2$ using AFM microscopy for each sample, the TCPP-functionalized graphene surface exhibited nearly three times higher exosome density (2.1 particles/ μm^2) compared to the PBASE-functionalized surface (0.7 particles/ μm^2). This observation aligned with the device response data for exosome detection in buffer.

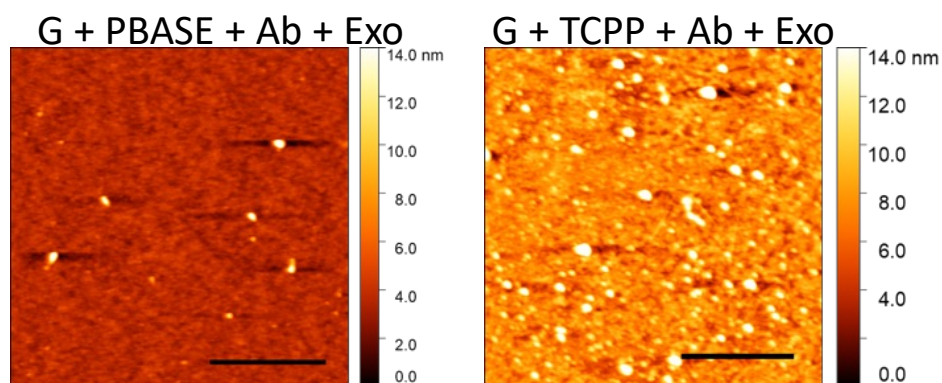


Figure 5-17 Atomic force microscopy (AFM) profile for characterization of exosome expression after 15 min incubation of 10^7 particles/ μL exosomes on PBASE modified graphene with antibody biofunctionalization and TCPP modified graphene with antibody biofunctionalization. AFM scale bars = $1 \mu\text{m}$.

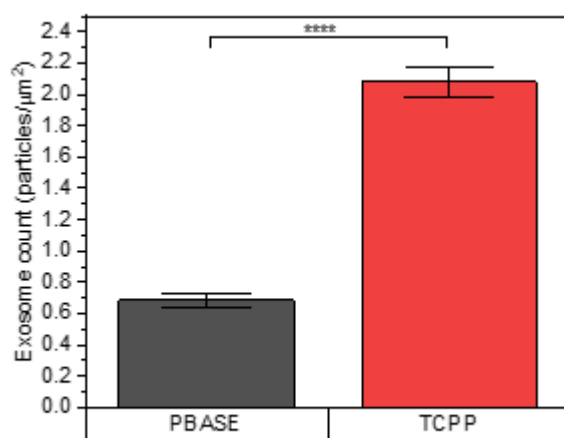


Figure 5-18 Exosome count on GFET sensors taken from an average of five spots on each of the functionalized graphene surfaces incubated with exosomes. (**** $P < 0.0001$). Data mean $\pm$ SD.

Based on the analysis of detected particles on the AFM images, an average increase in height of about 17.8 ± 8.0 nm after exosome incubation is extracted. (Figure 5-19) The measured mean height is consistent with the previous reported height distributions of exosomes characterized by AFM.

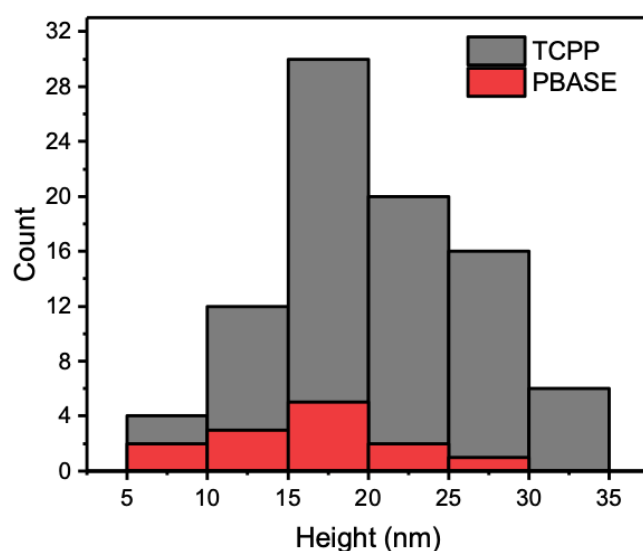


Figure 5-19 Histograms of height of exosomes as measured by AFM of PBASE and TCPP functionalized graphene with immobilized antibody after exosome incubation. The data were obtained on average from the analysis of around five $5 \times 5 \mu\text{m}^2$ AFM images.

5.5.3 Confocal microscope results

To further corroborate this finding, another independent method, confocal fluorescent microscopy, was employed to visualize the particles counts on both PBASE- and TCPP-functionalized graphene surfaces (Figure 5-20). The results were found to be consistent with those from AFM imaging. Specifically, the TCPP-modified graphene surface had three times the number of exosomes (62.5 particles/sample) compared to the PBASE-modified surface (17.4 particles/per sample) as depicted in Figure 5-21.

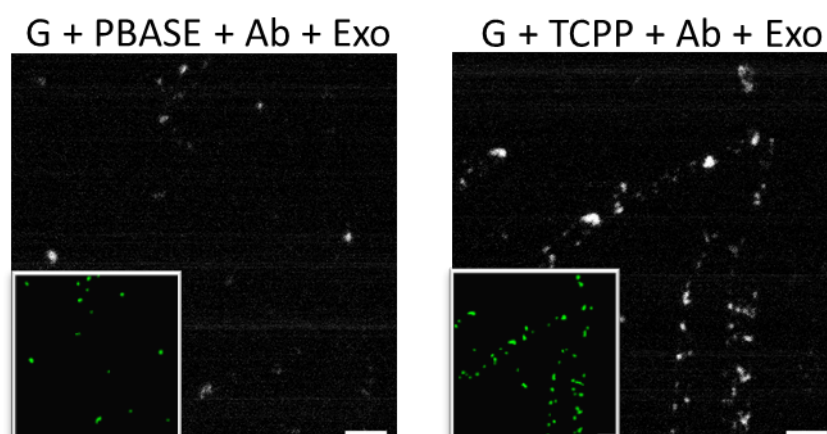
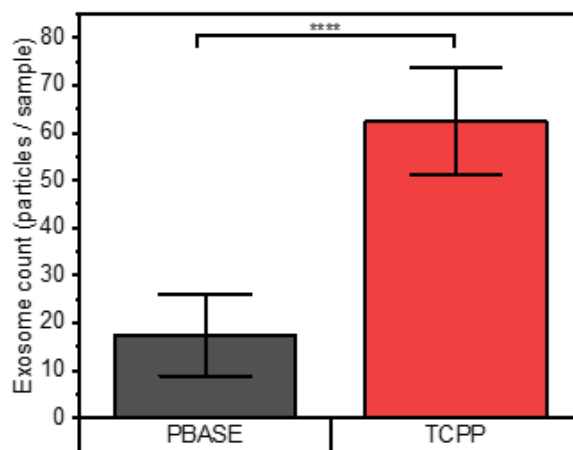


Figure 5-20 PBASE and TCPP Functionalized graphene surface with immobilized antibodies incubated with spiked fluorescent exosomes in PBS buffer. Exosome signal is shown in grey scale, and the insert shows the same field of view with the detected exosomes after bioimage analysis in green to make it clearer. Confocal scale bars = $5\mu\text{m}$.



*Figure 5-21 Fluorescence confocal microscopy images of exosome count on TCPP and PBASE functionalized graphene, taken from an average of five different spots on the TCPP modified graphene and five different spots on the PBASE modified graphene. (For details of the method used to count exosomes, see “Materials and Methods”). (**** $P < 0.0001$).*

5.6 Comparative performance for the detection of GPC1 using GFETs with PBASE and TCPP with clinical samples

To demonstrate the capability of the GFET biosensors in detecting disease biomarkers in real samples, clinical testing using the developed GFET reading platform was conducted. This involved analysing clinical blood plasma from two pancreatic cancer (PC) patients and two healthy controls. Glypican-1 (GPC-1) antibody was utilized as a biomarker detect cancerous exosomes with the GFET biosensors. Specific proteins are expressed at higher level on exosomes derived from pancreatic cancer cells compared to those from healthy ones. Such proteins can serve as biomarkers to diagnose and distinguish PC patients. Among these, GPC-1 is one of the proteins that is enriched on cancer-cell-derived exosomes and is highly expressed in exosomes from patients with PC, suggesting its potential as biomarker for PC detection.[20], [283], [284], [291], [292]

5.6.1 AFM

After the successful immobilization of GPC-1 antibodies, plasma samples were tested using both PBASE- and TCPP-functionalized GFET sensors. Firstly, nano-flow cytometry measurements were performed to quantify the concentrations of GPC-1+ exosomes in the plasma samples from the pancreatic cancer patients and the healthy controls. The GPC-1+ exosome concentration in the patient sample (1.18×10^{10} particles/mL) was significantly higher than in the healthy control sample (4.54×10^8 particles/mL). Then, microscopic imaging was performed to determine the exosome densities on surface-immobilized antibodies of both PBASE- and TCPP-functionalized graphene sensors. AFM was then executed on the GFET biosensors that was tested with clinical plasma samples. (Figure 5-22)

Figure 5-23 summarizes the performance of the PBASE- and TCPP-functionalized graphene with surface-immobilized GPC-1 antibodies in capturing and differentiating pancreatic cancer from healthy exosomes in the blood plasma samples. The results indicate that both the PBASE- and TCPP-functionalized GFET biosensors have substantially greater PC exosome coverage in comparison to healthy exosomes. This confirms the ability of both PBASE- and TCPP-functionalized GFETs as sensors for the detecting of GPC-1+ exosomes in plasma samples. Analysing a total scanned areas of $100 \mu\text{m}^2$ for each sample, it was discerned that the average expression level of pancreatic cancerous exosomes was threefold higher on the TCPP-functionalized graphene surface compared to the PBASE-modified graphene surface.

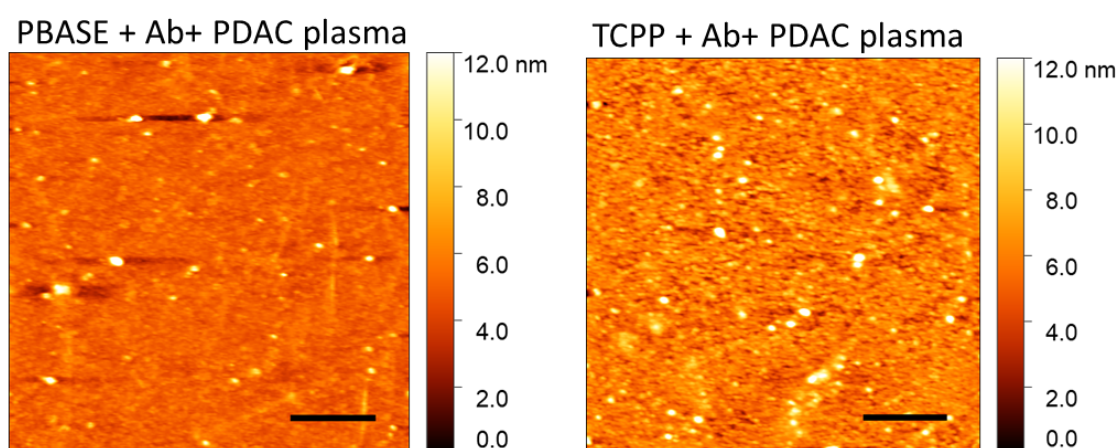


Figure 5-22 Characterization of surface functionalization of the GFET biosensor. (A) AFM profile of exosome expression on PBASE- and TCPP-functionalized graphene substrates immobilized with GPC-1 antibodies after 15 min incubation with PDAC patient plasma. AFM scale bars = 1 μm .

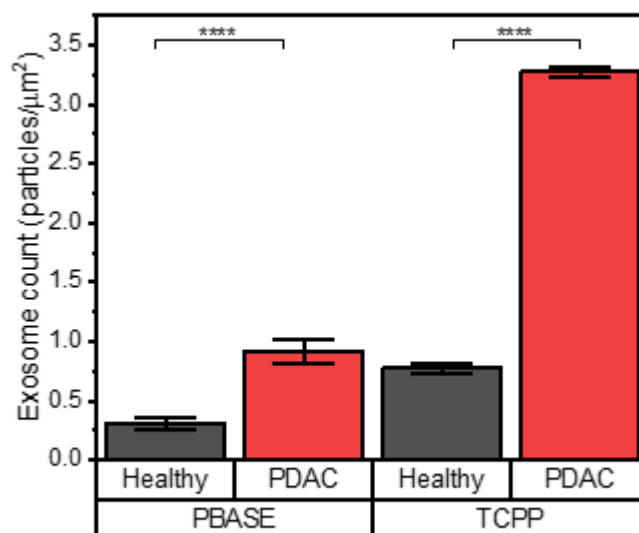


Figure 5-23 Numbers of particles captured by GPC-1 antibodies immobilized on 25 μm^2 of PBASE- and TCPP-functionalized graphene surface incubation with healthy and PDAC patient plasma for 20 min (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, $n = 3$). Data are mean $\pm$ SD.

5.6.2 Electrical performance

Measurements on the pancreatic cancer patient plasma and healthy plasma samples using the developed GFET reading platform were then performed. To facilitate testing with the undiluted plasma samples, a standardized testing protocol was implemented. This includes separate incubation and testing steps, complemented by multiple controlled rinsing steps (Materials and Methods). Figure 5-24 shows the representative transfer characteristic curves of the PBASE- and TCPP-functionalized GFETs before and after the incubating patient samples on the surface of the graphene.

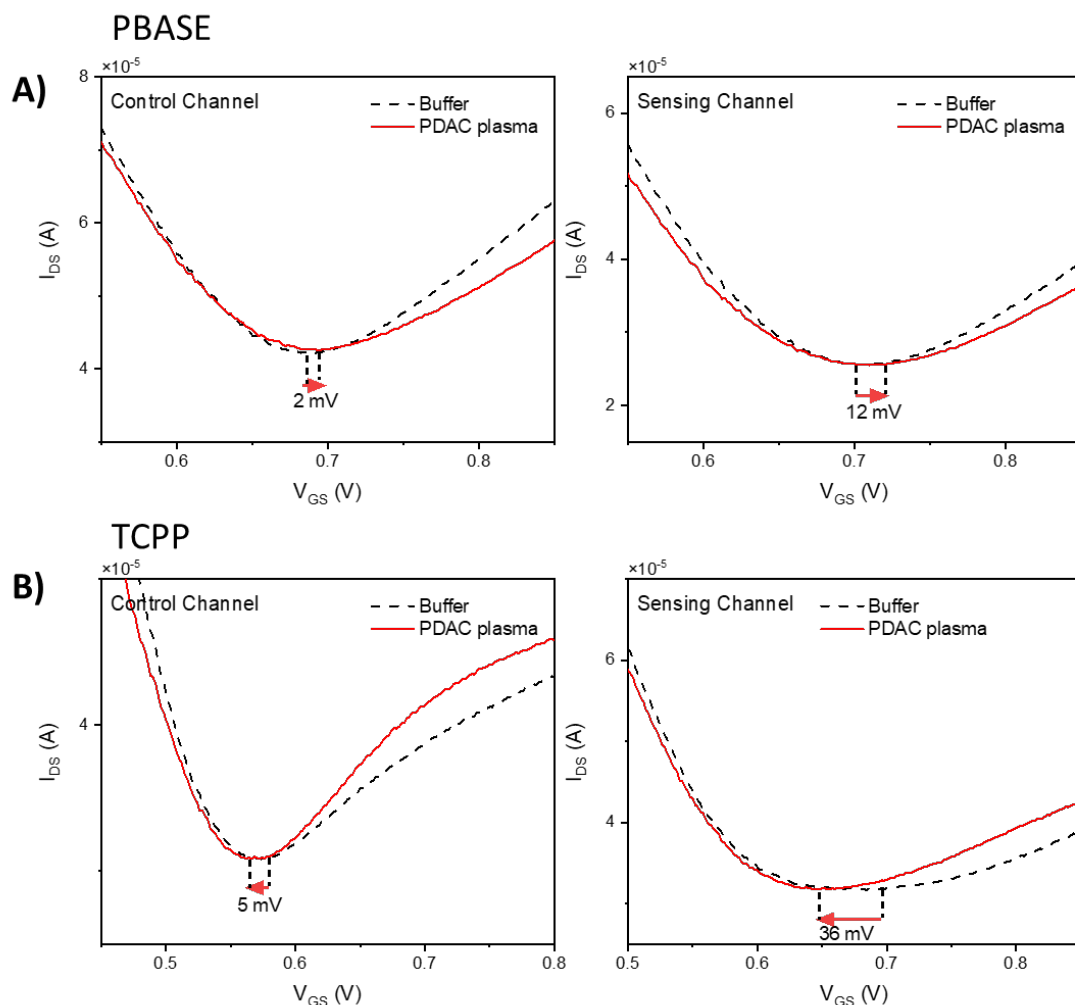


Figure 5-24 Detection of cancer exosomes in PDAC patient plasma using the GFET biosensor. Representative I_{DS} - V_{GS} curve of (A) control channel and (B) sensing channel on the GFET biosensor after exosome binding. A significant curve shift is seen in the sensing channel in comparison to the control channel, suggesting that the shift arises from exosome binding on the sensing channel.

As summarized in Figure 5-25, the significant difference in device responses to the plasma samples demonstrates the capability of the developed GFET biosensors to discriminate between PC patients and healthy controls. The detection response of the TCPP-functionalized GFET biosensors was stronger than that from the PBASE-functionalized sensors, which is consistent with the findings from the AFM study conducted on plasma samples from both Pancreatic ductal adenocarcinoma (PDAC) patient and healthy control. Notably, the sensitivity value of the TCPP-functionalized GFET biosensors was over three times that of the

PBASE sensors. These results validate the enhancement of GFET sensitivity achieved by modulating the orientation of surface-immobilized antibodies. The optimized orientation of antibodies on TCPP-functionalized GFET biosensors results in a higher binding probability, which in turn yields a more robust sensor response and higher detection sensitivity.

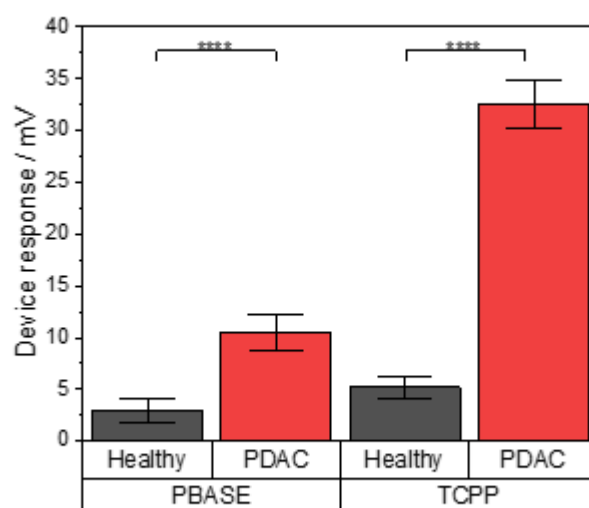


Figure 5-25 The measurement results for the GFET biosensors with 2 healthy plasma and 2 PDAC patients plasma illustrate the excellent sensitivity and selectivity of the TCPP-functionalized GFET biosensor (**** $P < 0.0001$, $n = 6$). Data are mean $\pm$ standard deviation (SD).

5.7 Summary

A biofunctionalization technique for graphene using the TCPP linker was developed, which enables the appropriate orientation of antibodies on the graphene surface. Through this method, an enhancement of two orders of magnitude in the LoD of GFET biosensors for detecting exosome biomarkers was achieved, compared with GFETs functionalized with the commonly used PBASE linker. Additionally, it is observed that over a threefold increase in exosome density on the TCPP-functionalized graphene surface relative to the PBASE-functionalized surface. The efficacy of the developed functionalization technique was also demonstrated by employing the developed GFETs to detect pancreatic cancerous exosomes

in patient plasma samples. The TCPP biofunctionalization approach holds significant promise for advancing ultrasensitive graphene biosensors. This method has the potential to enable highly sensitive and reliable detection of disease biomarkers on the graphene surface in real medium at rapid scanning rates. In the next chapter, the TCPP functionalization of GFETs will be integrated in the GFETs biosensors to validate of its performance in the detection of pancreatic cancerous exosomes in 26 clinical samples.

6 Clinical detection of PDAC cancer exosome in patients

6.1 Key Novelty and Achievements

After developing the GFETT platform, this Chapter aims to validate the performance of the GFET platform with clinical samples from 26 PDAC patients and healthy controls.

Although the ultra-sensitivity of graphene-based biosensors are reported in many papers, these results are mostly conducted in lab with buffer and filtered biomarkers. Real clinical samples are often with high salinity and complex biological composition. This will lead to many challenges, including Debye screening and false positive signals, which strongly hold back the development of graphene-based biosensors in testing with real clinical samples. Moreover, the reproducibility and device yield are other important factors that prevent wider clinical application with GFETs. In this Chapter, three unique designs are employed: Firstly, the use of differential measurements to compensate device drift and minimize false-positive signals. Secondly, the use of a standardized testing protocol with separated incubation and testing steps for Debye screening reduction. Lastly, the exclusion criterion is set based on more than 400 GFET devices tested in lab and applied this criterion to all clinically tested devices to ensure the device reproducibility and reliability. These designs lead to the success of the clinical trial presented in this chapter and give the novelty of the developed GFET sensors. The performance of the GFET sensor is characterized and demonstrated by the uniformity test, sensitivity test and selectivity test.

It is also worth mentioning that unique characteristics of cancerous exosomes and the higher density of GPC-1 proteins expressed on cancerous exosomes compared to healthy ones—which are confirmed in this Chapter—make the electrical detection using GFETs is an ideal platform to distinguish between pancreatic cancer exosomes and healthy ones when other technologies could produce a high level of false negative.

The portable electronic device was designed by Dr. Bruno Gil Rosa. The Clinical Samples are provided by Nabeel Merali and Dr. Adam E. Frampton. Part of the NanoFCM measurements is performed by NanoFCM Inc. as a service. Graphenea fabricated GFETs devices are used for the clinical testing.

The work presented in this Chapter is part of the Journal publication: “Yin, T., Xu, L., Bruno Gil Rosa, Nabeel Merali, Sokolikova, M.S., Gaboriau, D., Liu, D., Nizamuddin, A., Alodan, S., Chen, M., Oihana Txoperena, María Arrastua, Juan Manuel Gómez, Nerea Ontoso, Elicegui, M., Torres, E., Li, D., Mattevi, C., Frampton, A.E. and Jiao, L.R. (2023). Graphene Sensor Arrays for Rapid and Accurate Detection of Pancreatic Cancer Exosomes in Patients’ Blood Plasma Samples. *ACS Nano*, 17(15), pp.14619–14631. doi:<https://doi.org/10.1021/acsnano.3c01812>.”

6.2 Overview

Pancreatic cancer (PC) is the second most deadly cancerous disease and the seventh-leading cause of cancer deaths worldwide, with 5-year survival rates estimated to be below 7.3% and approaching zero for patients with advanced pancreatic cancer.[293] Despite the recent progress in cancer diagnosis and treatment, the overall 5-year survival rate in the case of PC has only marginally improved over the past decade. [294], [295] A critical factor in the poor outcomes is the lack of early symptoms. Most patients with symptoms present for a medical evaluation only when the cancer is at an advanced stage. However, the likelihood of survival can be significantly improved if the cancer is detected at an early stage. Therefore, early detection and diagnosis are crucial for improving outcomes.

Screening programs for PC can result in decreased incidence and mortality. Imaging techniques such as computed tomography (CT) and magnetic resonance imaging/cholangiopancreatography (MRI/MRCP) are primary methods used for the detection and evaluation of pancreatic tumours in clinics. However, the use of such methods to screen the general population for PC is not practical or cost-effective due to its low incidence rate. Recently, liquid biopsies that use novel biomarkers such as circulating tumour DNA (ctDNA),[296], [297], [298] circulating tumour cells (CTCs),[299] exosomes,[300], [301] and microRNAs (miRNAs)[302], [303] presented in body fluids have emerged as promising approaches for early PC diagnostics. The role of exosomes is discussed in detail in Chapter 5.3. There is increasing evidence that protein markers on exosomal pancreatic cancer-initiating cells (PCICs) are promising for the early detection of PC. Previous studies have reported that GPC-1 is specifically enriched on cancer-cell-derived exosomes.[287], [304] It has been found that PC patients express higher levels of GPC-1 on their exosomes than healthy controls with high specificity and sensitivity for PC.[291] Other exosome-based proteins such as epidermal growth factor receptor (EGFR)⁴ and epithelial cellular adhesion molecule (EpCAM)[305] also show high accuracy and selectivity for PC detection.

Current methods to detect specific exosomes, such as the Western blot, enzyme-linked immunosorbent assay (ELISA),[306] and flow cytometry, [307] usually take long time to complete from sampling to results, and require complicated processing steps. In recent years, many sensor technologies have been developed to enhance the limit of detection (LOD) of

assays for exosome detection, including potentiometric sensors,[308] electrochemical methods,[309], [310], [311] capacitive sensors,[312] fluorescence methods,[313] and surface-enhanced Raman scattering.[313], [314] There are only a few studies that specifically detect exosomal biomarkers from pancreatic patients' samples.[314], [315], [316] However, existing methods still lack the specificity and capability to detect early stage of cancer. Therefore, a direct, accurate, and highly sensitive diagnosis platform with the capability for the specific detection of pancreatic cancer in clinical samples at early stage is urgently needed. Here, an on-chip-based POCT (point-of-care testing) GFET sensor platform for the detection of pancreatic cancerous exosomes in patient plasma is developed (Figure 6-1). The platform consists of GFET sensors arrays with liquid gate electrodes integrated on the chip. A portable read-in/out electronic system was built to measure the real-time electrical response from the GFET sensors for 12 channels on one chip simultaneously. This platform is used to detect GPC-1 in plasma from 26 patients using a 20 μ L drop within 45 min. The GFET technology is clearly shown to be able to discriminate between samples from healthy controls and PDAC patients. A significant increase in the cancerous exosome binding to the sensor surface compared with healthy ones, even when they were both at the same volume concentration level in the plasma is observed. The GFET results are compared with MRI and CT data to evaluate the performance of the proposed GFET sensor for clinical testing. Furthermore, this platform is portable and can be easily integrated to detect simultaneously multiple cancerous biomarkers in real time. Thus, electrical detection using GFETs could be a promising diagnosis platform for the early diagnosis of pancreatic cancer and other diseases.

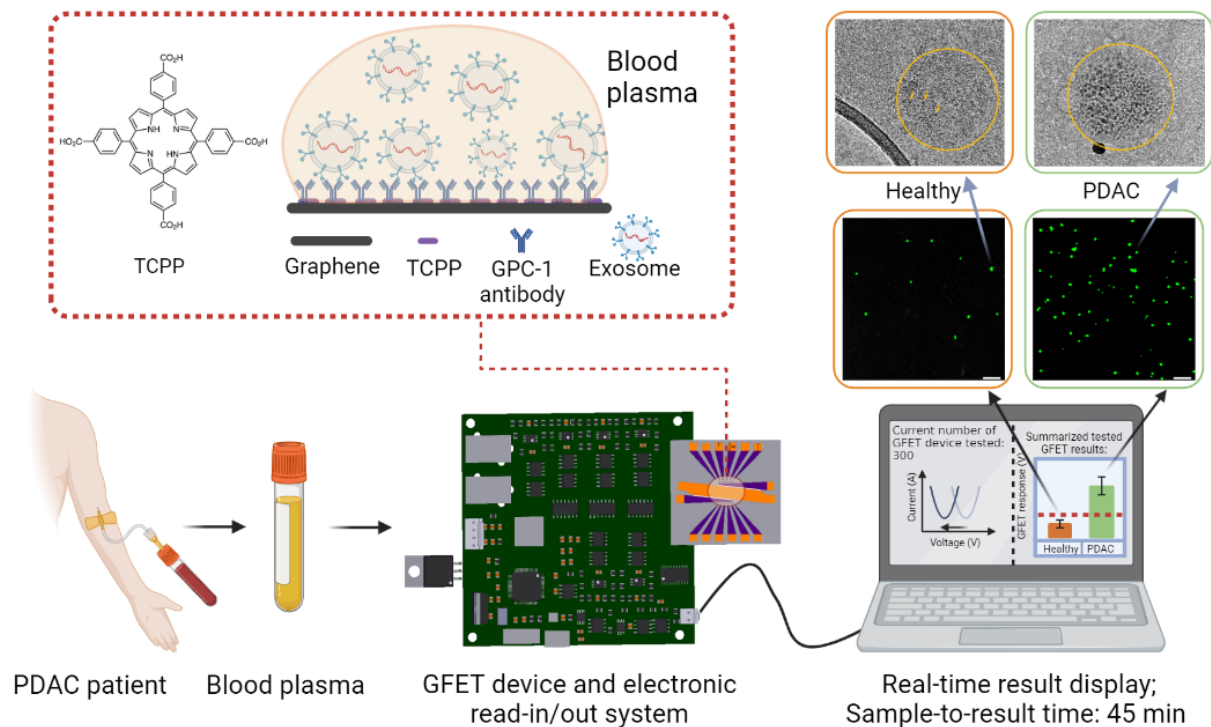


Figure 6-1. Schematics of detection of PDAC exosomes using GFETs with portable electronics and real-time detection results. The total detection time from applying blood plasma on the GFETs to results is less than 45 min. The zoomed in area shows schematics of the functionalization of graphene with TCPP [317] and the GPC-1 antibody. The middle-right images are fluorescence images showing higher density of exosomes on the GFET surface for the PDAC patients' sample than the healthy controls'. The top-right images are TEM images with immunogold labelling with GPC-1 to compare the GPC-1 proteins expressions on healthy and cancerous exosomes.

6.3 Fabrication and characterization of GFET biosensor

Although a high yield GFETs were fabricated in the cleanroom, for clinical samples foundry based GFETs chips were used to ensure chip-to-chip uniformity and consistency in the electrical reading.

6.3.1 Electrical properties

Electrical characterisations of the fabricated GFETs were performed to ensure the quality control.

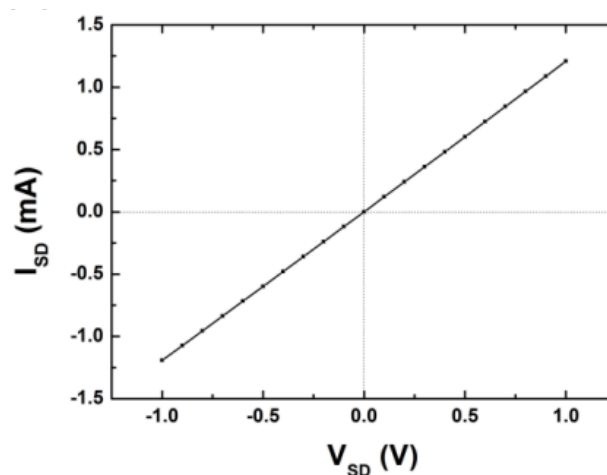


Figure 6-2 Typical output curve of the GFETs chips measured at room temperature. The output characteristics of GFET exhibits linear $I_d - V_d$ dependence.

The typical transfer curves of the GFETs with liquid gating is shown in Figure 6-3. The results obtained with both external Ag/AgCl electrode, and the on-chip electrode are shown.

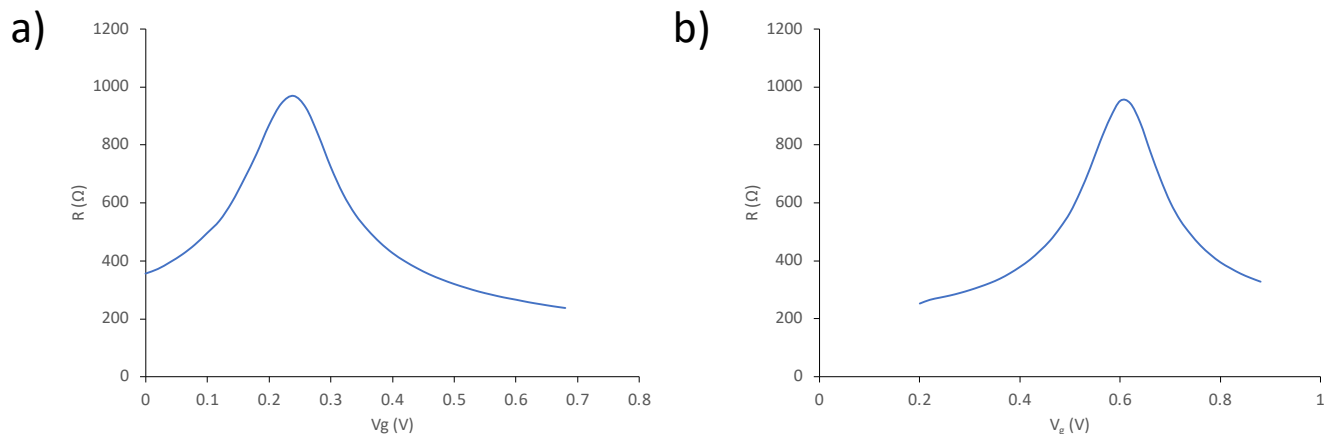


Figure 6-3 Typical transfer curve of the GFET chips with liquid gating, where the resistance of the device is measured as a function of the gate voltage V_g . a) An external Ag/AgCl electrode is used with the PBS buffer solution. b) The on-chip Au electrode is used with the PBS buffer solution.

In order to ensure the quality control of the GFET chips for the detection and to minimize the drift from GFET devices, preliminary tests in the GFET devices prior to the functionalization

was performed. The quality control measurements is based on 5 continuous sweep of the GFET devices, as shown in Figure 6-4. The slight drift of the device usually vanishes after the first five measurements and the transfer curve becomes stable. The Dirac point of the tested GFET device was extracted (Figure 6-5), devices with stable Dirac point are selected and used for functionalization and further testing. Electrical measurements with further buffer solutions or samples are performed after 5 measurements with the PBS buffer solution until the stability of the device is achieved.

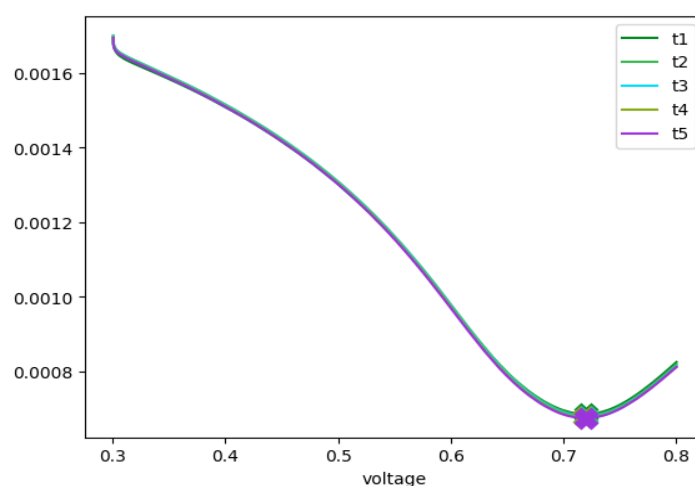


Figure 6-4 Representative I_{DS} - V_{GS} curve of the bare GFET chips tested in the PBS buffer solution for 5 continuous sweep readings prior to the functionalization steps for quality control.

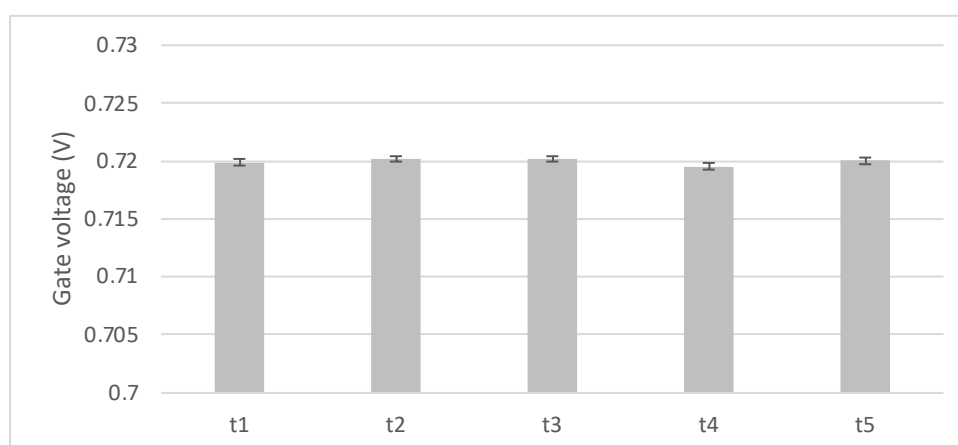


Figure 6-5 Example of the 5 continuous readings of the Dirac point of the GFET devices tested in the PBS buffer solution, illustrating low drift and stability of the GFET biosensor.

Various characterization techniques were performed to examine the functionalization of TCP and antibody on the graphene surface.

6.3.2 Raman spectroscopy measurements

The (Figure 6-6) recorded for bare graphene showed peaks at 1580 cm^{-1} , which is commonly attributed to the G band of sp^2 carbon in graphene. In addition, the presence of peaks at 1350 cm^{-1} (D band), 2700 cm^{-1} (2D secondary D band) is also shown, indicating a high quality of monolayer graphene. Raman spectroscopy analysis of the graphene surface after functionalization with TCP showed the characteristic peaks of TCP [318], which are the vibrations of C-N (1240 cm^{-1}), C-H (1454 cm^{-1}), C-C (1565 cm^{-1}), and a weak shoulder peak (1496 cm^{-1}). The Raman D-to-G peak of the graphene increased from 0.32 (bare graphene) to 0.89 (after functionalization with TCP), indicating that there is some disorder in the graphene sheet. The intensity ratio $I_{2D}/I_G = 1.89$ indicates high-quality graphene after the functionalization process.

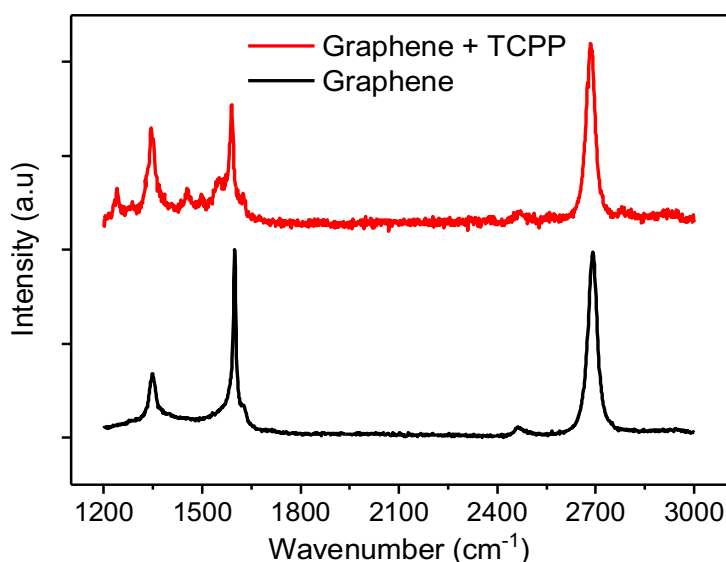


Figure 6-6 Characterization of surface functionalization of the GFET biosensor. Raman spectra of bare graphene and TCP-functionalized graphene.

6.3.3 X-ray photoelectron spectroscopy (XPS) measurements

X-ray photoelectron spectroscopy (XPS) was used to confirm the existence of TCPP and antibody on the graphene surface. GPC-1 antibodies were selected as a pancreatic cancer exosome biomarker targeting GPC-1 proteins on the surface of cancer exosomes. Figure 6-7 shows the changes in principal C 1s and N 1s core levels after each functionalization step. The high-resolution N 1s spectra show a significant increase in the N 1s peak at 399.8 eV after GPC-1 antibody conjugation. The high-resolution C 1s spectrum is composed of 4 components at the surface, C–C at 284.9 eV, C–O at 285.9 eV, C–N at 286.4 eV, and O–C=O at 287.7 eV, corresponding to the large number of amine and amide bonds present in the antibodies.

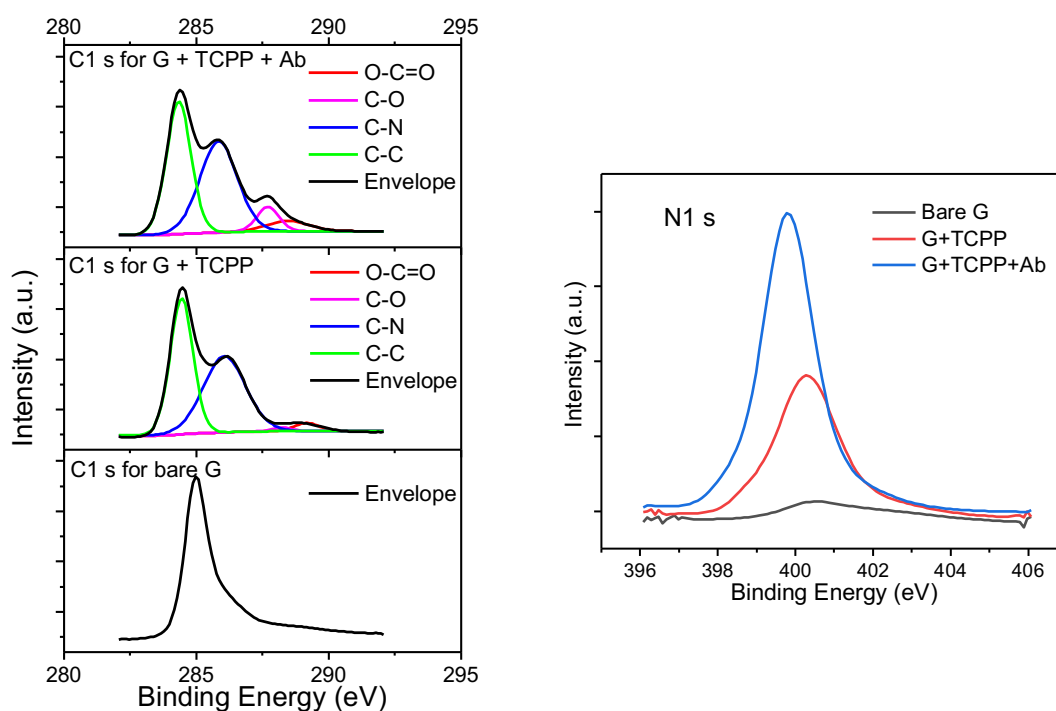


Figure 6-7 Characterization of surface functionalization of the GFET biosensor. C1 s and N1 s X-ray photoelectron spectroscopy (XPS) spectra of graphene with different degrees of functionalization: bare, with TCPP, and with TCPP + antibody respectively.

6.4 Clinical Cohorts

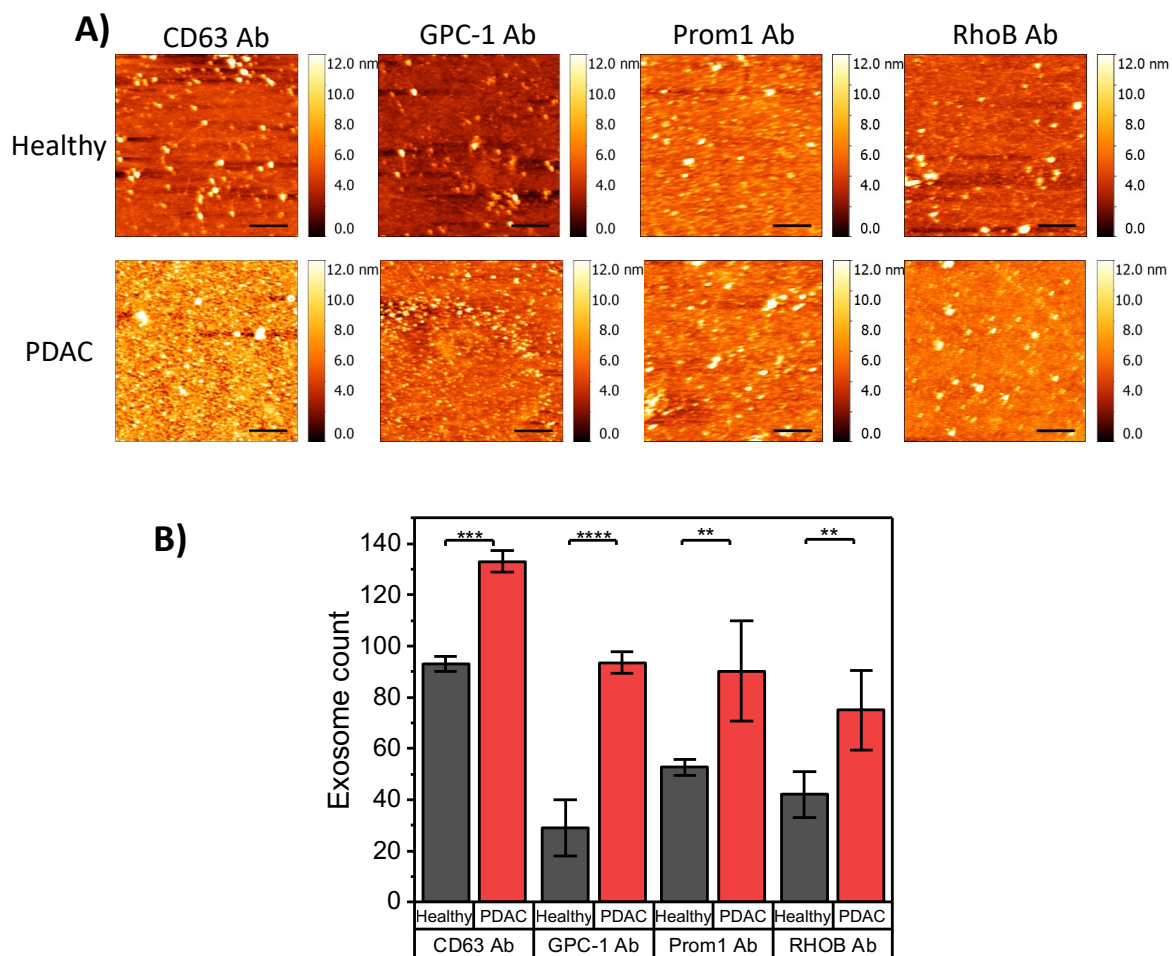
Details of the patient cohort is summarized in Table 6-1.

Table 6-1 Summary of the patient cohort.

	PDAC group	Healthy control group
Number of patients	18	8
Age-yrs.		
Median	69	69.5
Range	49 - 84	60 - 74.5
BMI (kg/m²)	24.64	n/a
Gender		
Male	10 (55.6%)	4 (50%)
Female	8 (44.4%)	4 (50%)
Smoking	8 (44.4%)	2 (25%)
Tumour size – mm		
Median	27.5	n/a
Range	15 - 38	n/a
Diagnosis		
PDAC Stage	no. (%)	Benign
I	1 (5.56%)	gallstones, n=3
II	1 (5.56%)	gallstone pancreatitis, n=1
III	7 (38.9%)	duodenal diverticulum, n=1
IV	7 (38.9%)	duodenal mucosal cyst, n=1
V	1 (5.56%)	Papilla fibrosis, n=1
Metastatic	1 (5.56%)	fatty infiltration of pancreas, n=1

6.5 Selection of antibody for PDAC cancer exosome detection

Exosomes are extracellular vesicles that carry proteins, nucleic acids, and lipids. Many exosome-enriched proteins have been reported. Specific exosomal enriched proteins are expressed more on pancreatic cancer cell-derived exosomes compared to healthy ones, which offers the possibility to diagnose and distinguish patients with PC.[287], [291], [304] Protein biomarkers enriched on pancreatic cancer-cell-derived-exosomes include glypican-1 (GPC-1),[287] RHOB,[319] CD63 and Prom1.[320] All of these proteins can be expressed on both cancerous and healthy exosomes.¹⁴ In order to choose an appropriate biomarker for detection using GFETs, the protein should be highly expressed on cancerous exosomes with low expression on healthy exosomes. Therefore, the selectivity of each protein biomarker to cancer cell-derived compared to healthy exosomes was tested. Four types of antibodies (CD63, GPC-1, Prom1, and RhoB) were immobilized on the graphene surface to investigate their sensitivity and selectivity to PDAC cancer exosomes in patient plasma samples. Figure 6-8 summarizes the performance of these antibodies in differentiating PDAC cancer exosomes from healthy exosomes in blood plasma. Using AFM, it was found that all protein biomarkers are abundant on the PDAC cancerous exosomes. All the selected protein biomarkers were found to have increased expression in plasma exosomes in PDAC patients compared to the healthy controls. Furthermore, CD63 antibodies exhibited the highest capture rate for both cancerous and healthy exosomes. These results were expected, because CD63 has been observed to be enriched on all exosomal membranes.[321], [322] The high concentration of CD63+ exosomes confirmed the presence of exosomes in the tested samples.[115], [321] We found that Prom1 antibodies have low selectivity, while that of RHOB was higher. However, we also found that the GPC-1 antibodies had the highest selectivity to the PDAC cancer exosomes and the lowest capture number of healthy exosomes (Figure 6-8 B), showing significantly higher specificity to the PDAC cancer exosomes. The specificity of each protein for cancer and healthy exosomes were 1.4:1, 3.2:1, 1.6:1, and 1.8:1 for CD63, GPC-1, Prom1 and ROHB respectively. These results confirm that the GPC-1 antibody is suitable for the detection of PDAC cancer exosomes[304] and it was selected to be used for conjugation onto the GFET surface in the clinical testing.



*Figure 6-8 Selection and validation of antibodies for the clinical detection of pancreatic cancer cell-derived exosomes in patient plasma: A) AFM profile of exosome expression on 4 different antibodies functionalized on TCPP decorated graphene substrate (CD63, GPC-1, Prom1, and RhoB antibodies) after 15 min incubation with healthy plasma and PDAC patient plasma. AFM scale bars = 1 μ m. B) Numbers of particles captured by CD63, GPC-1, Prom1, and RhoB antibodies functionalized on 25 μ m² of graphene surface after 20 min incubation with healthy plasma and PDAC patient plasma (** P < 0.01, *** P < 0.001, **** P < 0.0001, n = 4). Data are mean $\pm$ s.d.*

6.6 Design and Optimization of the performance of GFET sensor array

Although it has been demonstrated that single GFETs are able to detect biomarkers at femtomolar concentration under laboratory conditions,[323], [324], [325] the level of consistency of the GFET response for a larger number of sensors under the condition of a clinical study has barely been reported yet. Bio-detections with GFET sensors are often limited

to measurements in non-physiological solutions with low ionic strength due to the Debye screening effect. To realize the detection in blood plasma, the analytical performance of the fabricated GFET biosensors was optimized for pancreatic cancer exosome detection *via* the application of an internal control channel and the development of an optimum test protocol. Furthermore, since overcoming the issues of reproducibility of GFETs fabrication and the uniformity of electrical response from device-to-device and chip-to chip are important in clinical testing. The use of foundry fabricated GFETs can help to overcome the issues of uniformity. In addition, an exclusion criteria before clinical testing to improve consistency and the reliability of detection is set up. (Figure 6-9) Moreover, the advantage of graphene arrays was emphasized for the reliable detection of ions,[326] aiming to overcome the shortcoming of the graphene technology. Here the necessity of GFET arrays and negative control subarrays for meaningful detection of exosome biomarkers in clinical samples from cancer patients are demonstrated.

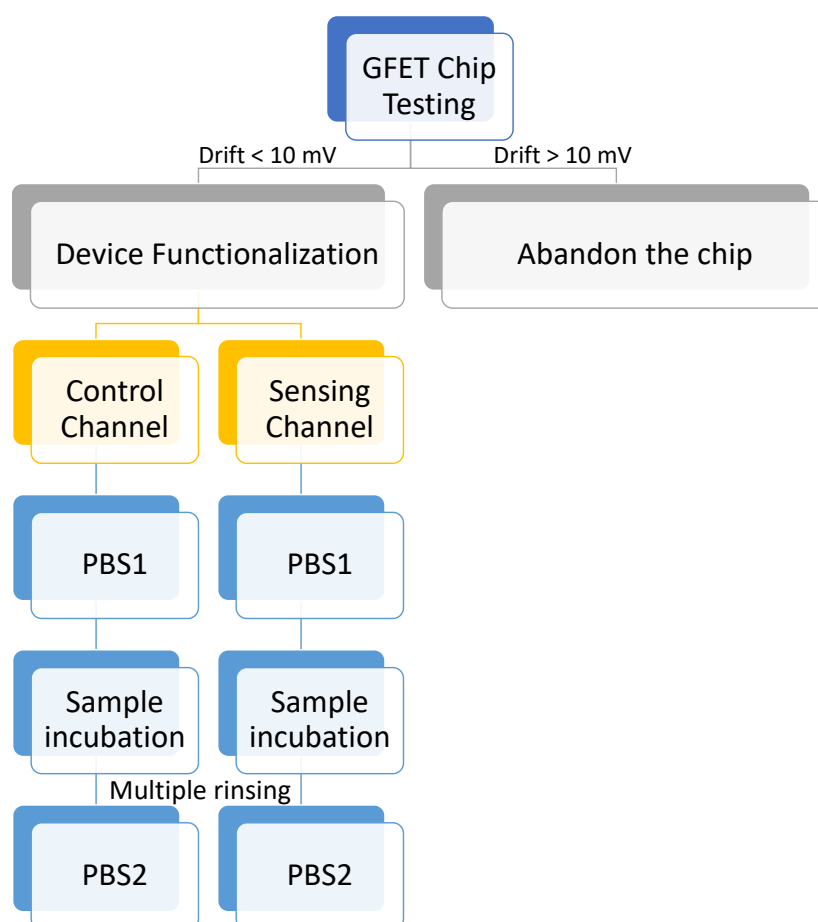


Figure 6-9 Schematic flow diagram of the protocol implemented for the GFET testing with the exclusion and inclusion criteria applied.

The fabricated GFET biosensor is an on-chip integrated sensor array (10 mm × 10 mm) comprising 12 individual GFET devices with a graphene channel area of 100 μm × 100 μm and a window opening of 90 μm × 90 μm deposited between the source and drain electrodes. These devices are separated into two sections by the central common-on-chip integrated Au electrode. The on-chip integrated liquid gate allows the simultaneous measurement of all 12 GFET devices for the detection of the same sample solution (Figure 6-10).

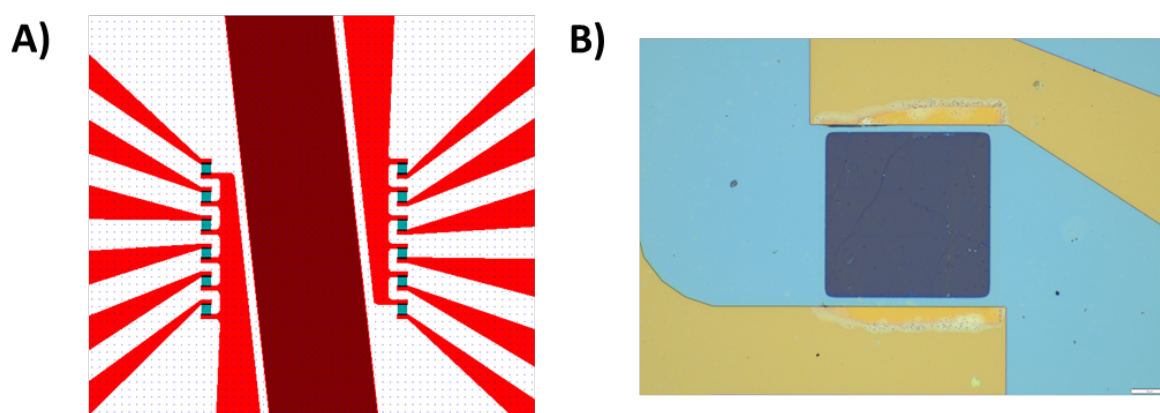


Figure 6-10 A) Illustration of the layout of on-chip integrated GFET sensor, designed with 6 devices each on the left and right sides. These devices are separated by the central common-on-chip integrated Au electrode. B) Illustration of the optical image of on-chip integrated GFET devices.

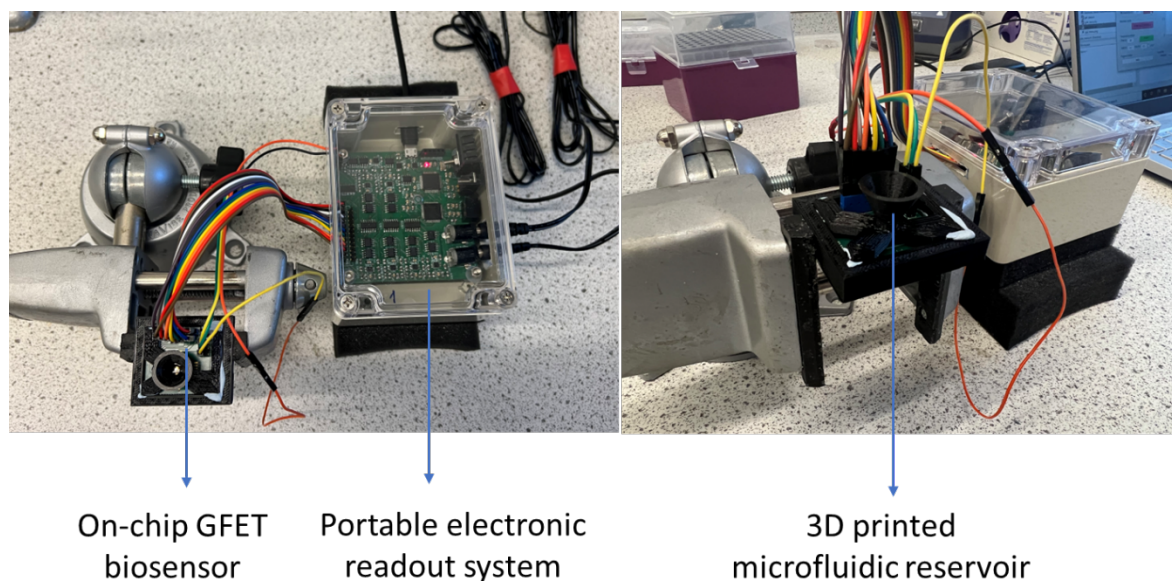


Figure 6-11 Illustration of the graphene chip with the 3D printed microfluidic reservoir, and PCB board-based portable electronics. Top view : left, side view : right.

Due to the complex physiological environment of blood plasma with high concentration of other non-desired species, the direct detection with blood plasma is very challenging. The noise produced by non-specific binding interactions will lead to a reduction in selectivity and limiting detection sensitivity. Therefore, two separate sections of the devices are classified as a sensing channel (6 GFETs devices in parallel on the left-hand side) and a control channel (6 GFETs devices in parallel on the right-hand side). The sensing channels are functionalized with GPC-1 antibodies for specific target detection in sample solution, whereas the control channels lack any antibody functionalization in aiming for the detection of background signals in the same sample solution (Figure 6-12). The use of control and sensing channels can help in distinguishing between signals arising from specific target binding and interfering signals caused by background buffer or unwanted non-specific binding events on the graphene surface. Hence, the accuracy and reliability of the detection of specific targets can be improved.

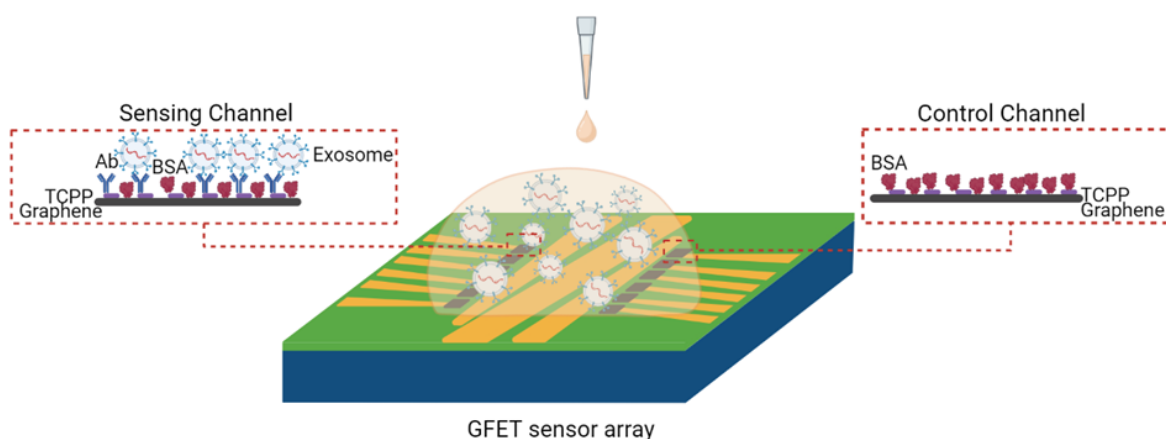


Figure 6-12 Schematics of the on-chip integrated GFET biosensor platform with the antibody functionalized channel as the sensing channel and a channel without antibody functionalization as the control channel.

All liquid measurements with the GFET sensor array are performed with an in-house designed and fabricated microfluidic channel (Figure 6-11), as introduced in section 3.6.4. This microfluidic channel is specifically designed as a reservoir with fixed position for all fabricated chips. The use of the fabricated microfluidic channel can improve the performance of the GFET sensors.

In addition, one of the critical aspects of biosensor is device uniformity and reproducibility. The uniformity and reproducibility of the GFET sensor performance were investigated by comparing the GFETs response for five chips to the same concentration of spiked target exosomes (10 ng/ μ L) in buffer. The change in Dirac voltage remains at 13.48 ± 0.71 mV with 5% chip to chip variation as shown in Figure 6-13. Furthermore, the GFET devices on each chip show a small device-to-device variation. The average change in Dirac voltage was within 2%. Therefore, the repeatability and the uniformity in electrical response from chip-to-chip and device-to-device is satisfactory for clinical testing is found.

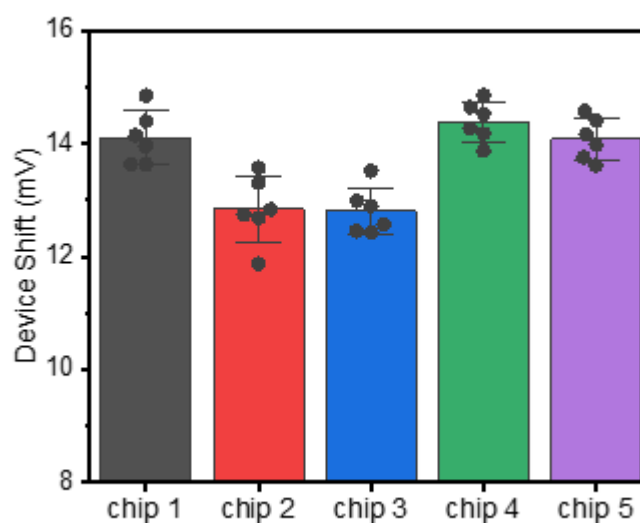


Figure 6-13 GFET device detection signal comparison between the five chips tested for the same concentration of exosomes in buffer, illustrating low device-to-device variation and high chip-to-chip reproducibility of the GFET biosensor. Error bars are determined by the standard deviation of multiple device measurements, $4 \leq n \leq 6$ for each reading.

An optimized test protocol was also implemented which includes a separated incubation and testing steps. Tests of clinical samples included multiple rinsing and electrical testing steps prior to the injection of undiluted plasma samples onto the GFET chip. As the GFET biosensor's response is strongly affected by the ionic strength of the solution, the chips were rinsed with PBS after 15 mins incubation of the samples. 15 minutes of incubation is selected, previous literature [286] has reported that the GPC-1 exosome level in healthy plasma can be $< 10^8$ particle/ μL which is supported by the average level of healthy exosome in plasma measured in this study. Based on a diffusion model developed in earlier study, [115] it was found that it takes about 15 mins to accumulate 2 exosomes/ μm^2 on graphene surface. This number of exosomes is the number required to achieve a measurable signal by GFETs based on an analytical model that is recently developed which gives the surface potential at graphene surface due to exosome binding. The model will be discussed in details in the following section. It is worth noting that the 15 mins incubation time is not a fixed time. It depends on several factors including the target type, target concentrations, the binding kinetics regime. This time

was optimized based on the expertise and previous work on exosomes, their concentrations in healthy and plasma samples, and analytical models. A few iterations were performed in the lab first to optimize the incubation time for this clinical study. The incubation time can be greatly reduced, for example, if the convection transport effect is added that can allow more exosomes to be collected at the sensor surface. Further studies on the diffusion of exosomes to the surface of GFET sensors and its corresponding responses are introduced in Section 6.9.

In order to remove the false positive signals arise from the highly viscous plasma residue on the surface of graphene, the amount and number of $\times 1000$ diluted PBS used to rinse after the sample incubation step is standardized to be $3\times$ times of $200\ \mu\text{L}$ of PBS each time. (Figure 6-14) All measurements were recorded in $\times 1000$ diluted PBS in order to reduce the screening effect. This should lead to improved sensing performance and reliability of the sensor response.

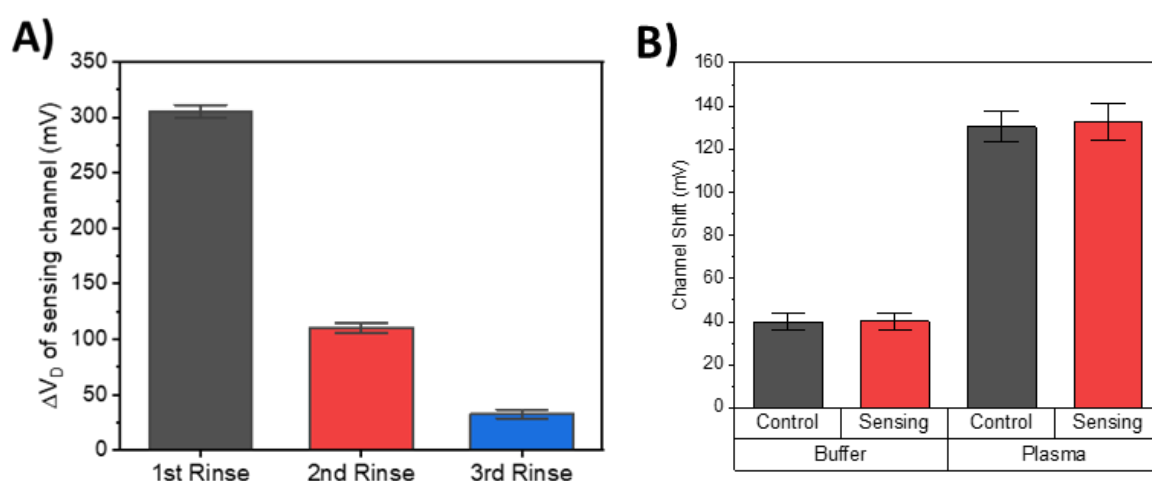


Figure 6-14A) Comparison of device shift from sensing channel with different times of rinse. The decrease in device shift is due to the complex environment of blood plasma samples. The high salinity and sticky nature of blood plasma led to salt accumulation and left unwanted residues on the surface of GFET sensors B) Comparison of channel shift in control and sensing channels when testing with pure buffer and healthy plasma sample.

The rinsing step is important to reduce the Debye screening from high ionic strength plasma sample. The decrease in device shift is also due to the complex environment of blood plasma samples. The high salinity and sticky nature of blood plasma led to salt accumulation and left unwanted residues on the surface of GFET sensors. In order to prove the specificity of

antibody based detection, testing in pure buffer was performed before the incubation and testing with plasma samples. It is shown in Figure 6-14 B) that both control and sensing channels have the same amount of shift when testing in buffer. While testing with healthy plasma samples, the shift in both sensing and control channels is significant, however the difference between these two channels is small. There is a slightly larger shift in the sensing channel due to the amount of GPC-1+ exosomes present in healthy plasma. Therefore, during the rinsing processes, the reduction in signal is mainly due to removal of surface accumulated salt and unwanted species. Moreover, AFM was performed on both the control channels and sensing channels of the tested GFET. On the surface of the control channels, there is no or only a few exosomes shown. While on the surface of the sensing channels with GPC-1 Ab immobilized, a significant number of exosomes is shown. (Figure 6-15) These suggested that the importance of rinsing and antibody based detection is verified.

It is worth noting that the channel shift observed in the control channel when testing in buffer is due to the drift of GFET devices. There are several reasons that could lead to the drift of GFETs in buffer, including ion redistribution after the application of an electric field, trapped ions between graphene and substrate, defects in graphene or interface traps in the substrate that can generate a false positive response and reduce the accuracy of detection. [53], [145], [230] Therefore, it is important to use internal control channel to minimize the effect of GFETs device drifts and reduce the background noise.

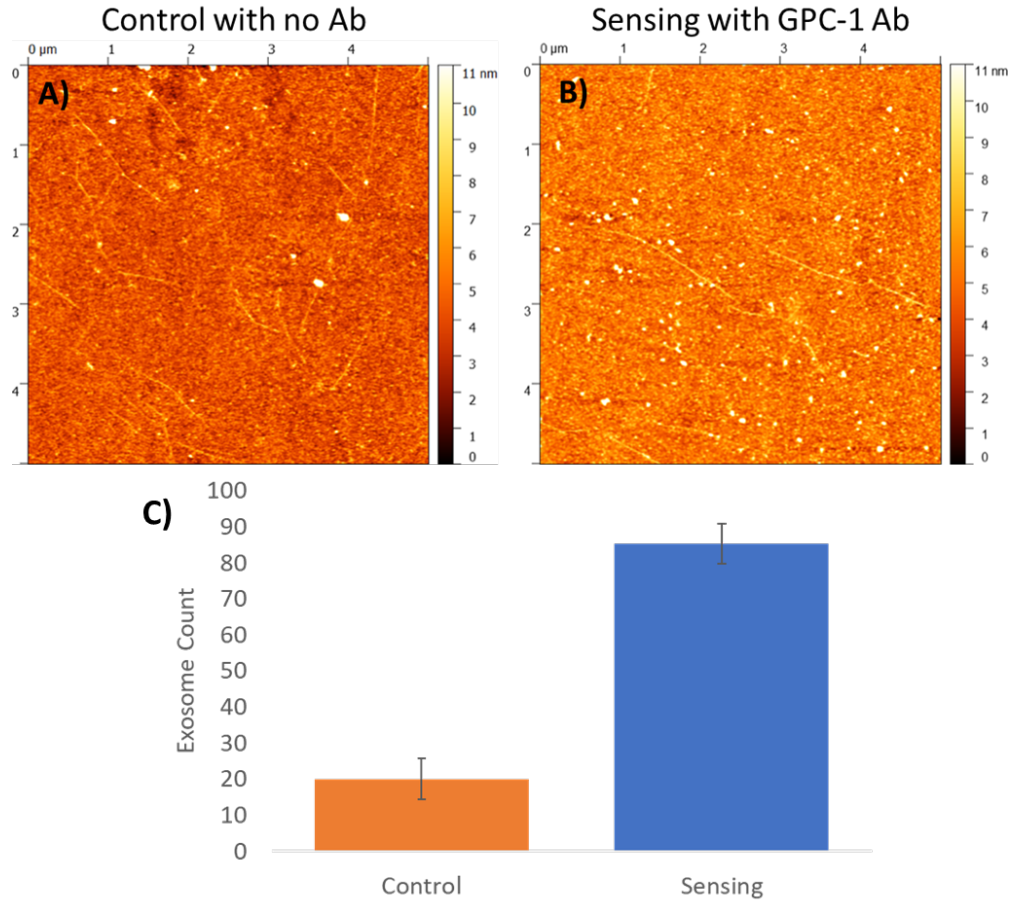


Figure 6-15 Atomic force microscopy images of A) representative control channel of developed GFET sensor after incubation and testing with plasma sample. B) representative sensing channel of the developed GFET sensor after incubation and testing with plasma sample. C) Exosome count on GFET sensors taken from an average of five spots on each of the functionalized graphene surfaces incubated with plasma samples. Data are mean $\pm$ standard deviation (s.d.).

Furthermore, in order to ensure the stability and reliability of the GFET devices during clinical testing, a device exclusion criteria was set up, which enabled highly reproducible and reliable results to be collected based on more than 440 laboratory-tested devices (Figure 6-16). The Dirac voltage for the lab-tested devices was $V_D = 0.71 \pm 0.24$ V. To eliminate the effect of device drift, devices with $V_D < 0.47$ V or $V_D > 0.95$ V were excluded in this study. The average resistance of the devices was measured in air at $R = 1230 \pm 400$ Ω , and therefore devices that exhibited values of $R > 1700$ Ω or $R < 700$ Ω were also excluded.

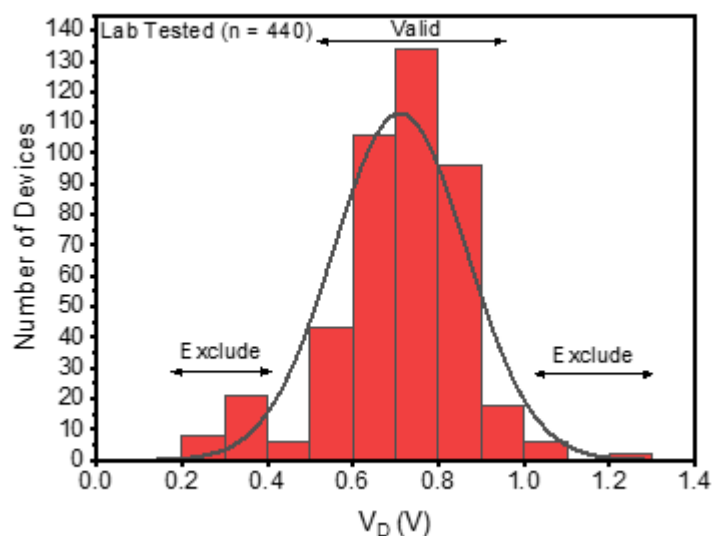


Figure 6-16 Device exclusion criteria for GFET reproducibility and validity. Histogram of Dirac voltage for lab-tested GFET devices with Gaussian distribution curve. Device exclusion criteria were set based on 444 lab-tested GFET devices, where the Dirac voltage for lab-tested devices was $V_D = 0.71 \pm 0.24$ V.

To evaluate the analytical performance of developed GFETs, transfer characteristic measurements was performed on GFET sensors for detection of spiked model exosomes in buffer. The characteristic I-V curves of the GFET biosensor upon detection of exosomes in PBS in concentrations from 10^{-1} ng/ μ L to 10^3 ng/ μ L were recorded. A significant left shift of the Dirac point with increased exosome concentration in comparison to the buffer solution is shown (Figure 6-17).

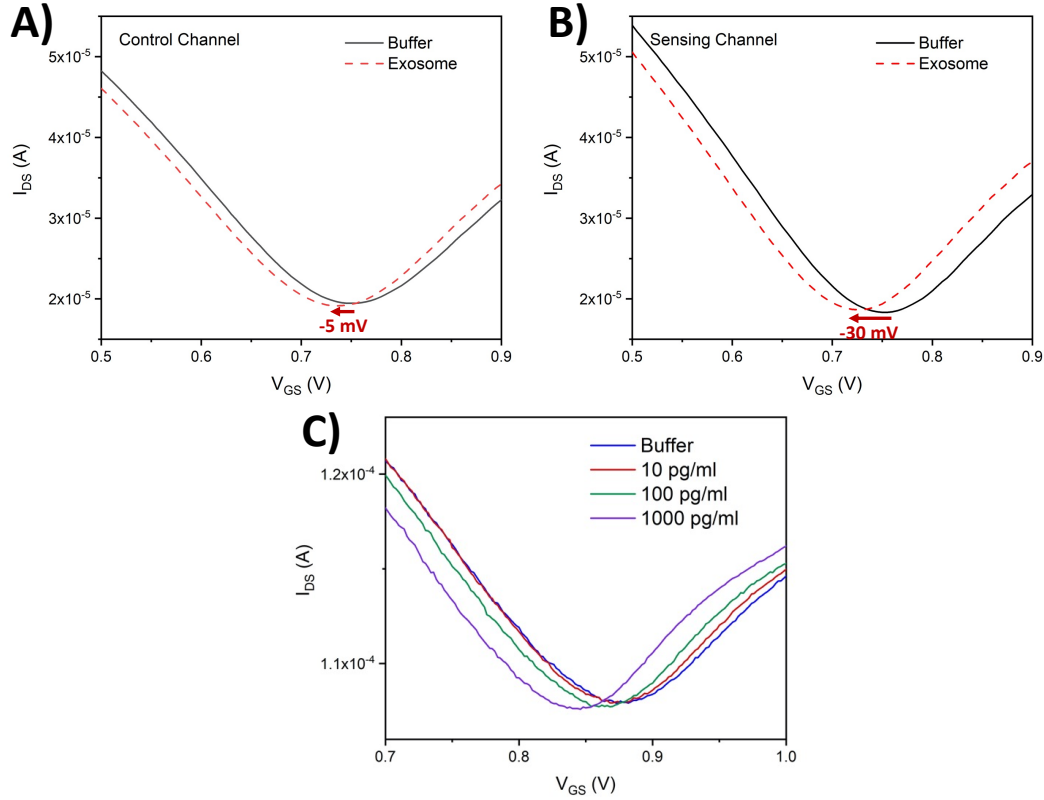


Figure 6-17 Validation of GFET biosensor for detection of exosome with protocol of using control and sensing channel. Detection of cancer exosomes in buffer using the GFET biosensor. Representative I_{DS} - V_{GS} curve of the A) control channel and B) sensing channel on the GFET biosensor after exosome binding. Significant curve shift seen in the sensing channel in comparison to the control channel, suggesting that the shift arise from exosome binding on sensing channel. C) Representative I_{DS} - V_{GS} curve of the sensing channel of GFET biosensor in buffer with various concentrations of exosomes.

Figure 6-18 shows the calibration curve of the change in Dirac voltage against target concentration ($y = 4.06 \log(x) + 9.68$; $R^2 = 0.9823$). A logarithmic dependence on spiked exosome concentration with the device shift is shown. This proves the excellent sensitivity of the GFET sensors.

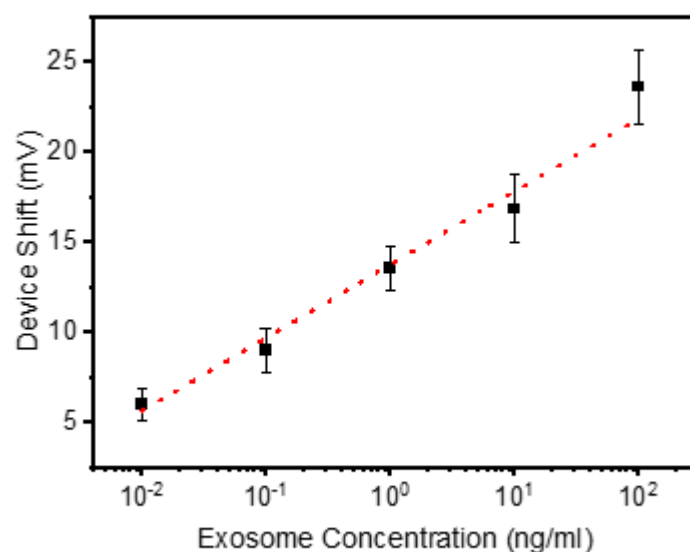


Figure 6-18 Calibration curve of the GFET biosensor for detection of various concentrations of model cancerous exosomes in buffer. Error bars are determined by the standard deviation of multiple device measurements, $4 \leq n \leq 24$ for each reading.

The selection of antibody is also crucial in achieving the high selectivity and sensitivity of biosensors. The GPC-1 protein is expressed in many cancer cell derived exosomes. It is shown in many literatures[287], [327], [328], [329] that there is an increase in the expression of GPC-1 for patient with pancreatic cancer. Many studies performed has demonstrated that GPC-1+ exosomes can be potential biomarker for the diagnosis of PDAC at its early stage. Therefore, in this study, the expression of GPC-1 on exosomes in blood plasma from PDAC patients was confirmed, as introduced in Section 6.5 and used it as a putative cancer marker for the detection of cancerous exosomes using the GFET biosensor. It was selected to be used for conjugation onto the GFET surface in the clinical testing.

In order to test the selectivity of GFETs for GPC-1+ cancerous exosomes prior to clinical testing, healthy plasma samples was spiked with model exosomes and measured the GFET responses with and without spiked exosomes. The measurement results are shown in Figure 6-19. The healthy control plasma induced a small signal of 3.5 ± 2.7 mV, which could be attributed to the small percentage of GPC-1 present in healthy plasma. There are around 0.3-4.7% GPC-1+

exosomes in healthy human plasma samples.[286] On the other hand, the addition of various concentrations of model cancerous exosomes (0.01 $\mu\text{g}/\mu\text{L}$, 0.1 $\mu\text{g}/\mu\text{L}$ and 1 $\mu\text{g}/\mu\text{L}$) caused a significant shift in Dirac voltage of 37 ± 7.9 mV. This indicates that the developed GFET biosensor can detect cancerous exosomes in blood plasma without any processing or preparation required. The detection results also demonstrate high selectivity of the GFET sensor to cancerous in comparison to healthy exosomes.

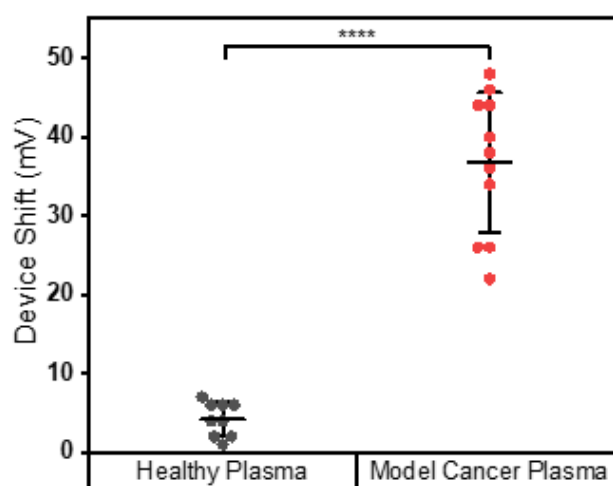


Figure 6-19 Measurement results for the GFET biosensor with healthy plasma and model cancer plasma, illustrating excellent selectivity of the GFET biosensor. (**** $P < 0.0001$). Data are mean $\pm$ standard deviation (s.d.).

6.7 Clinical detection of PDAC cancer patient samples with GFET

Next, the developed GFET biosensor was used for the detection of GPC-1+ exosomes in blood plasma with a cohort of 26 patients, including 18 cases of PDAC patients and 8 healthy controls (Table 6-1). Only a small droplet of 20 μL of each sample is required for testing with a GFET sensor. The internal control channel and BSA were used to reduce non-specific binding and false positives (Figure 6-20 A). GFETs transfer curves in buffer solution inherently drift due to several possible reasons, including ion redistribution after the application of an electric field, trapped ions between graphene and substrate, defects in graphene or interface traps in the substrate that can generate a false positive response and reduce the accuracy of

detection.[53], [145] Therefore, utilizing internal control is crucial to compensate and minimize the effects of the drifts and reduce the background noise.[230]

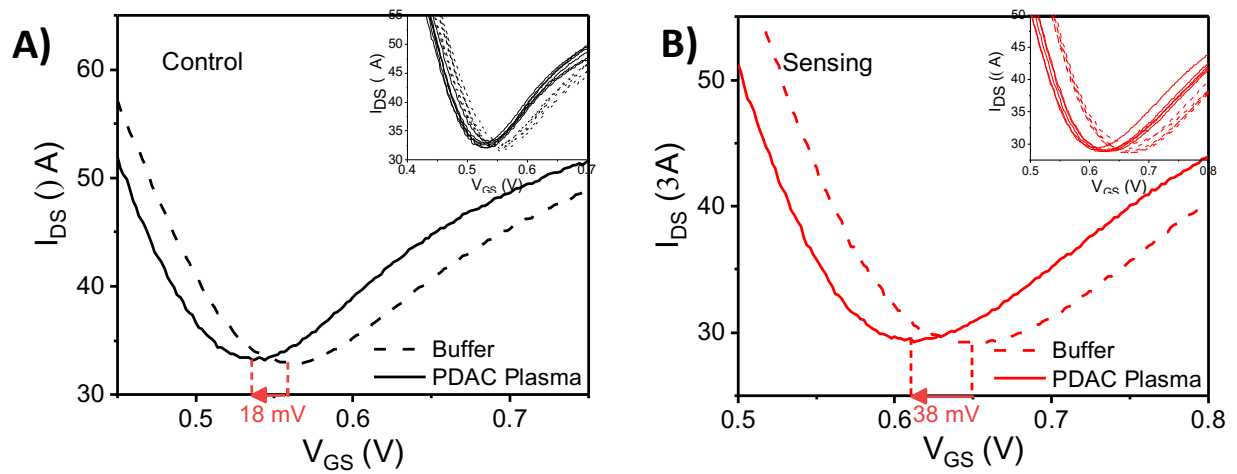


Figure 6-20 A) Representative I_{DS} - V_{GS} curve of one of the control channels on the GFET biosensor before and after PDAC plasma incubation. The inset image shows the response from 6 control channels on the GFET biosensor. B) Representative I_{DS} - V_{GS} curve of one of the sensing channels on the GFET biosensor before and after PDAC plasma incubation, showing a significant curve shift in the sensing channel.

Figure 6-20 B shows a representative graph of the GFET transfer curve before and after the incubation of a patient sample on the surface of graphene. The difference in Dirac voltage shift between sensing and control channels was used to determine the GFET response. (Figure 6-21)

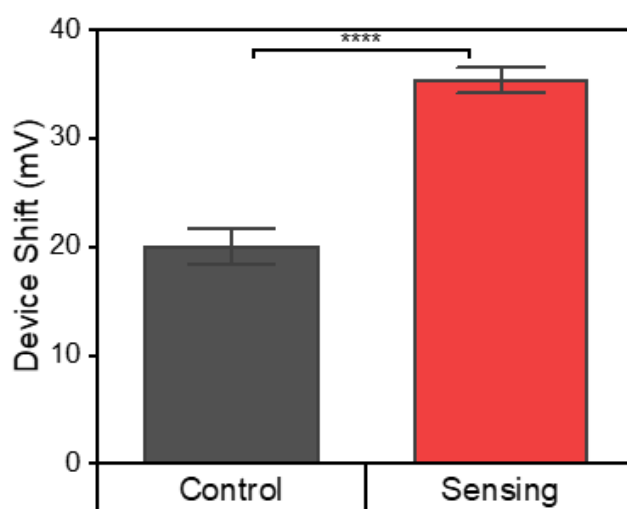


Figure 6-21 Device shift of the 6 control and 6 sensing channels on a representative clinically tested GFET device. The device shift arising from a plasma sample is calculated based on the difference between the GFET response from control and sensing channels.

The measurements before and after incubation were taken in $\times 1000$ diluted PBS to ensure strong coupling between the graphene and exosomes charges in the electrical double layer. The presence of 6 GFETs in the internal control and 6 in the sensing channels improves the statistical analysis and reduces detection error. Figure 6-20 A and B shows a representative response from the total of 12 channels, including 6 control and 6 sensing channels. Highly uniform and small device-to-device variations in response are usually observed (inset in Figure 6-20). The measurement results from the 26 patient samples are shown in Figure 6-22.

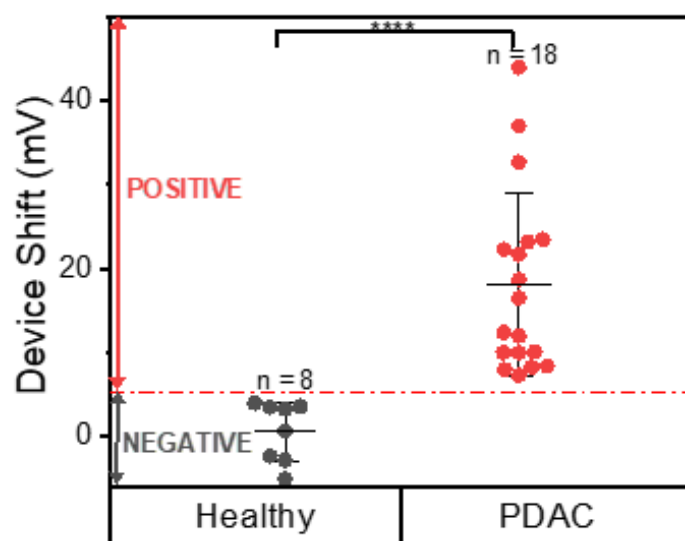


Figure 6-22 Measurement results for the GFET biosensor from the clinical testing with plasma samples from 18 PDAC patients and 8 healthy controls, indicating a clear threshold detection signal line which suggests that the sensor is able to differentiate PDAC patients from healthy controls (**** $P < 0.0001$). Data are mean $\pm$ standard deviation (s.d.).

Of all the devices tested, 281 GFETs satisfied the exclusion criteria and only the test results from these devices were included in the clinical testing (Figure 6-23).

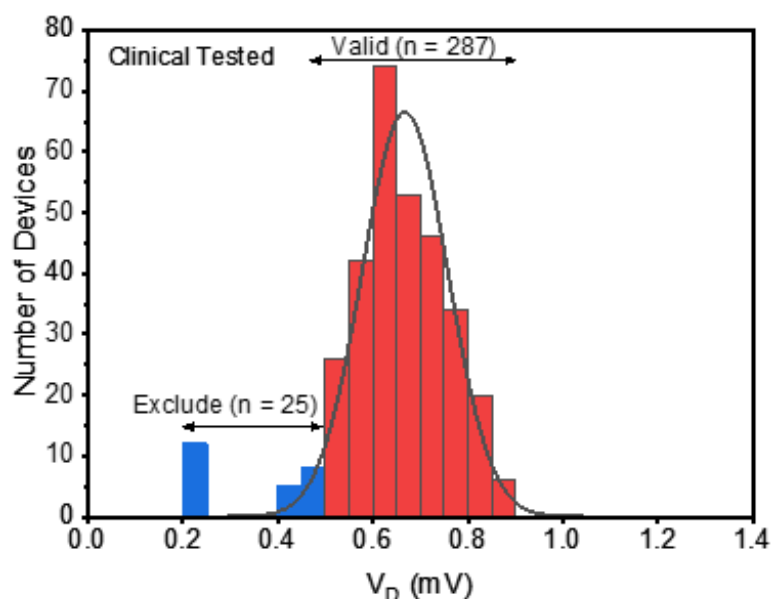


Figure 6-23 Device exclusion criteria for GFET reproducibility and validity. Results from $n = 281$ clinically tested devices that satisfied the exclusion criteria are included in the study. Devices ($n=25$) with $V_D < 0.47\text{ V}$ or $V_D > 0.95\text{ V}$ were excluded in this study. Devices ($n=12$) that exhibited values of $R > 1700\ \Omega$ or $R < 700\ \Omega$ were also excluded.

The developed GFET biosensor can clearly discriminate between PDAC patients and healthy controls, with a clear threshold line at 4.5 mV. (Figure 6-22 and Figure 6-24) There is an average shift in Dirac voltage with healthy control plasma of $0.5 \pm 3.5\text{ mV}$ (mean $\pm$ s.d.), whereas all PDAC patients showed a significant change in the Dirac point larger than 7.3 mV with an average shift of $18.0 \pm 10.9\text{ mV}$ (mean $\pm$ s.d.). This increase in GFET response could be a result of higher levels of GPC-1+ exosomes produced by tumorous cells in the PDAC patients' blood plasma.[286]

Principal component analysis plot of the relevant channel shift signal for PDAC and control group is performed. The performed principal component analysis showed that PC1 captured the greatest variance of the data set, (99.08%). It is shown from the PCA plot of channel shift for PDAC patient and healthy control that a clear separation line can be drawn on the PC1 of the PCA plot. This separation line can well separate the healthy control from the PDAC patient. The variance of the principal components, PC1, also indicated the device uniformity. This is because a more uniform device will result in less variation in the data set and therefore less variance in the principal components. These results suggested that the developed GFET biosensor is able to separate the PDAC patient from healthy control, with low chip to chip signal variation.

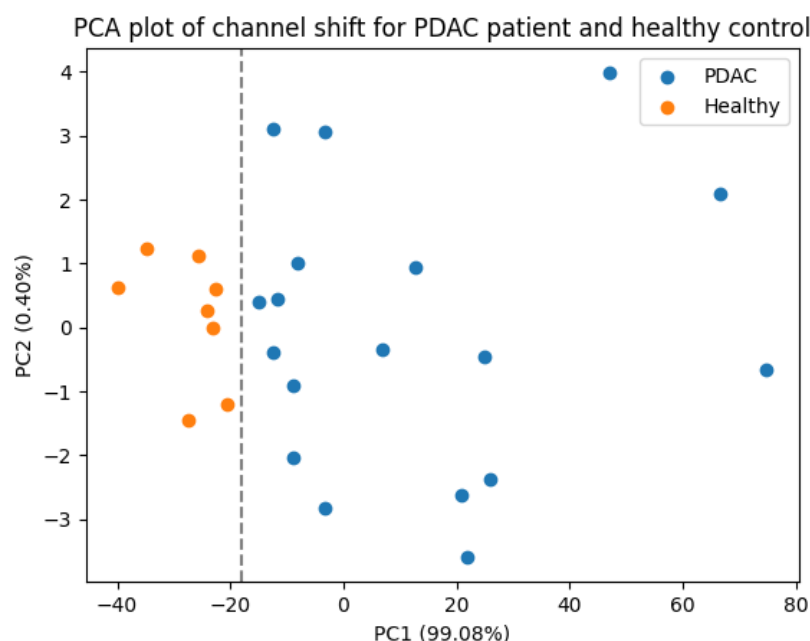


Figure 6-24 Principal component analysis (PCA) plot of the relevant channel shift signal for PDAC and control group. PC1 captured the greatest variance of the data set, (99.08%). A clear separation line can be drawn on the PC1 of the PCA plot. This separation line can well separate the healthy control from the PDAC patient.

6.8 Validation of GFET response to GPC-1 exosome concentration

In order to further validate the results, Nano Flow cytometry measurements^{[330][331]} is performed, a commonly used power analytical tool for biological nanoparticles like exosomes down to less than 100 nm diameter by light scatter, to determine the concentrations of GPC-1+ exosomes in the plasma samples from the healthy controls and PDAC patients tested.

6.8.1 Nano Flow Cytometry

Nano flow cytometry (Nano FCM) allows for size and concentration measurements of exosomes isolated from the blood plasma samples. Further subpopulation analysis by Nano FCM can be achieved by fluorescent (FL) labelling of targets. This fluorescent labelling can identify which particles are EVs using membrane or tetraspanin labelling. Meanwhile, through specific protein labelling, the present of surface markers, such as GPC-1, can also be identified.

All tested plasma samples (26 patient and 8 control) were analysed using Nano FCM for the identification of GPC-1+ exosomes in tested samples. The protocol of Nano FCM was optimised using 4 samples prior to the measurement with all samples. Then, prior to the ultracentrifugation to isolate the exosomes from blood plasma, samples were analysed unstained, to determine the concentrations of particles in blood plasma samples. The total number of particles in the blood plasma samples ranges from 1.61×10^{11} particles/mL to 4.76×10^{12} particles/mL. The mean sizes of the particles of samples are between 59.2 nm to 100 nm. The size distribution of two samples is shown in Figure 6-25.

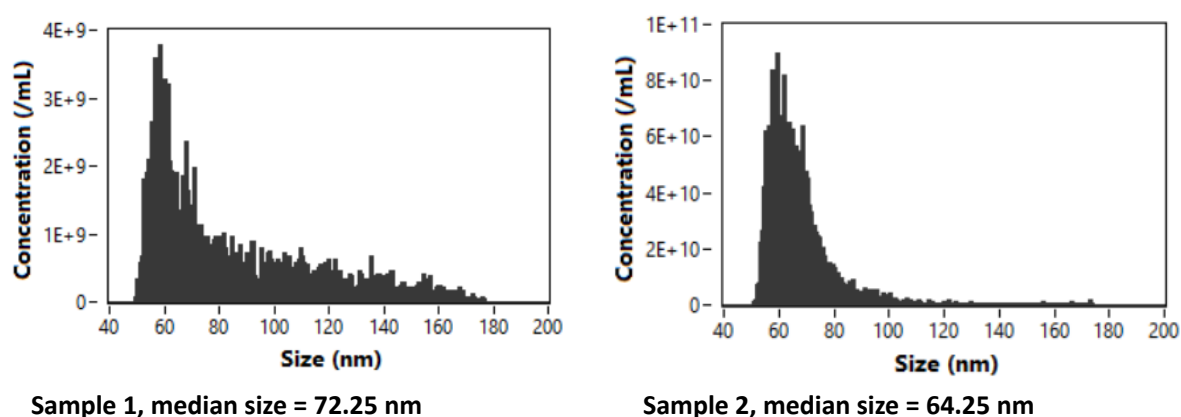


Figure 6-25 Example size distribution of particles in blood plasma samples prior to ultracentrifugation.

The samples were then labelled with high concentration of fluorescent anti-GPC-1 antibodies and ultracentrifuged. The stained samples were then measured using Nano FCM.

To ensure that the staining protocol is working, the fluorescent background of the samples were measured and analysed. If there is lots of free unbind antibody in the solution, the signal from fluorescent background will be high. A PBS control was run, in which 50 μ L of blank control PBS was stained with the fluorescent GPC-1 antibody and performed ultracentrifugation. The results are shown in Figure 6-26. In the PBS control (Figure 6-26 A), the intensity of the fluorescent background is ~ 300 . On the other hand, in the samples (Figure 6-26 A), the intensity of the fluorescent background is < 50 . This indicated that, when sample

particles are present in the solution, there is less free antibody present in solution. This showed that the antibody is able to bind to the fluorescent GPC-1 antibody.

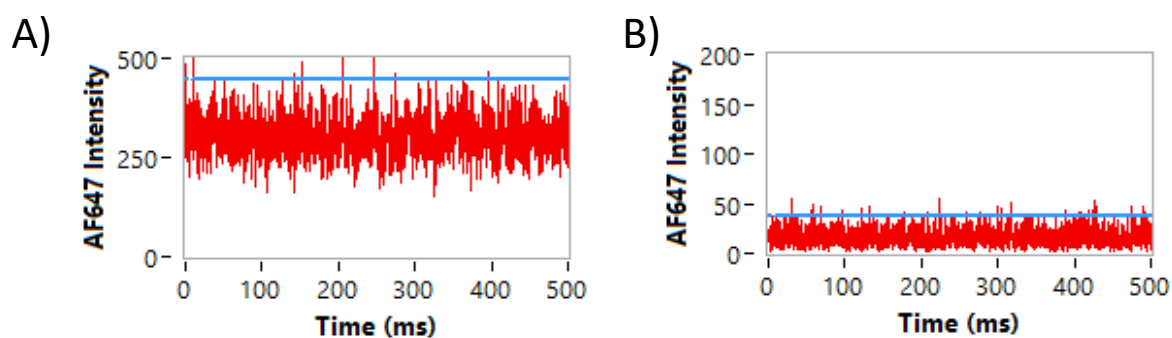


Figure 6-26 Fluorescent intensity of the background for A) control measurement with blank PBS and B) sample measurement.

Further measurement was also performed to ensure that the exosomes in the solution is saturated with GPC-1+ antibody labelling. Through diluting the samples with different serial dilution factors, the fluorescent baseline change was shown. Sample was diluted to both 1 in 100 (Figure 6-27 A) and 1 in 50 (Figure 6-27 B).

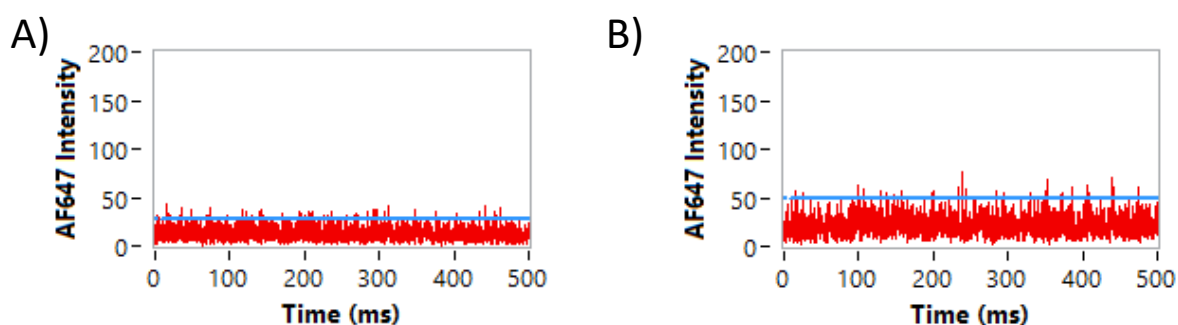


Figure 6-27 Fluorescent intensity recorded for samples with different serial dilution factors. A) Sample diluted to 1 in 100 and B) 1 in 50.

For the sample with the 1 in 50 dilution, the fluorescent baseline raised. This indicated that there is free antibody present in the solution. This result showed all the available exosomes with GPC-1 expression has been bound by the fluorescent anti-GPC-1 antibody, leaving free antibody left over during the ultracentrifugation process. This was observed for all measured

samples that were diluted to multiple dilution factors, showing that enough antibody was present during the staining procedure for Nano FCM measurements.

Figure 6-28 shows that GPC-1+ exosome concentrations were significantly higher in patient samples than in healthy ones, with average levels of GPC-1 found to be an order of magnitude higher in the former (range from 3.21×10^8 particles/mL to 2.11×10^{10} particles/mL, with a mean of 3.59×10^9 particles/mL) compared to the latter (range from 4.96×10^7 particles/mL to 5.13×10^8 particles/mL, with a mean of 2.18×10^8 particles/mL). The data obtained are in close agreement with that previously published in the literature,[286] indicating that the expression of GPC-1+ exosomes in blood plasma significantly increases in tumour-derived exosomes compared to healthy normal cells.

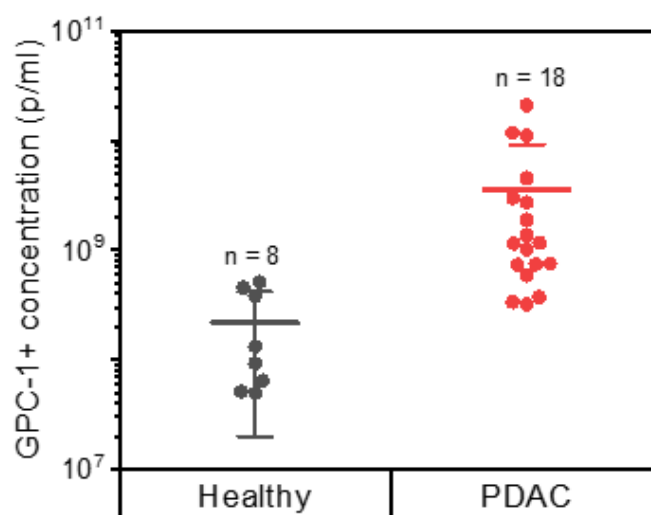


Figure 6-28 Validation the detection of PDAC cancer exosomes in PDAC patient and healthy control plasma. Concentrations of GPC-1+ exosomes detected in 18 PDAC patient plasma samples and 8 healthy controls plasma samples by Nano Flow cytometry measurements. Comparison of numbers of PDAC and healthy exosomes captured on GFET biosensors.

6.8.2 Surface characterisation techniques

Interestingly, three PDAC samples were found with concentrations of exosomes at similar levels as those in healthy samples, while the GFET responses were found to be twice as strong as for the healthy ones. Compared with healthy ones, cancerous exosomes have generally

been reported to carry more electrical charge at their surfaces, and this could contribute to the increase in the GFET response for PDAC samples.[332] However, this may not explain the significant increase in the sensor response for PDAC samples. Another possible explanation could be a high binding affinity between GPC-1+ and antibodies, which could result in a larger density of exosomes on the surface. Therefore, two fluorescence confocal microscopy experiments were performed on PDAC samples with a concentration of exosomes of 3.38×10^8 particles/mL and healthy samples with an exosome concentration level of 4.54×10^8 particles/mL. It found that the exosome density on the graphene surface was 54.5% higher for PDAC samples compared to healthy samples (

Figure 6-29 and Figure 6-30).

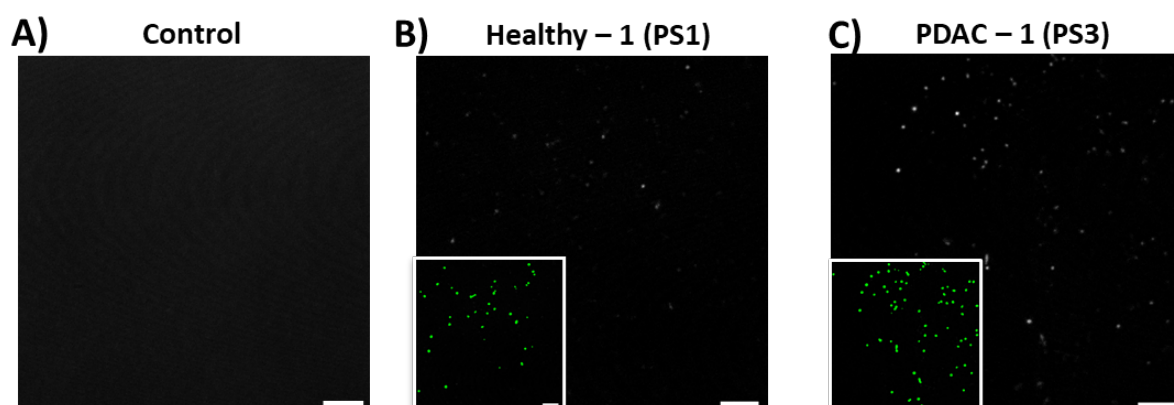


Figure 6-29 Fluorescence confocal microscopy images of exosome on GFET sensors. A) Functionalized graphene surface with no exosome as control. B) Functionalized graphene surface incubated with healthy plasma sample (PS1). C) Functionalized graphene surface incubated with PDAC plasma sample (PS3). Exosome signal is shown in grey scale, and the insert shows the same field of view with the detected exosomes after bioimage analysis in green' to make it clearer. Confocal scale bars = 5 μ m.

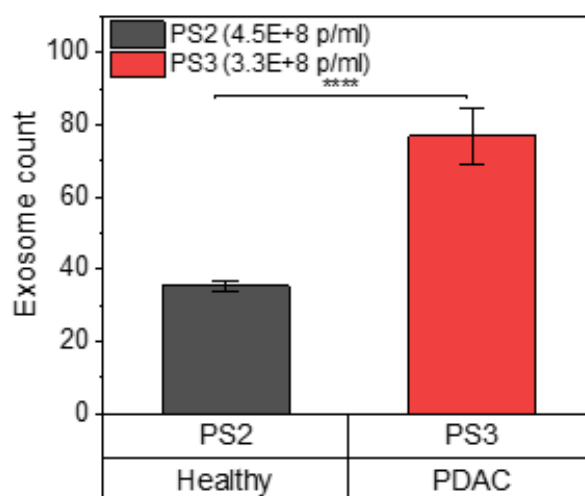


Figure 6-30 Fluorescence confocal microscopy images of exosome count on GFET sensors taken from an average of three positions on the functionalized graphene surface incubated with PDAC plasma sample and three positions incubated with healthy plasma sample (**** $P < 0.0001$).

In order to further validate this finding and to quantify the number of particles on the surface, other independent techniques, AFM, was used to image the number of particles on the surface (Figure 6-31 and Figure 6-32) and SEM (Figure 6-33 and Figure 6-34) on 2 PDAC and 2 healthy samples with similar levels of exosome concentration in the plasma.

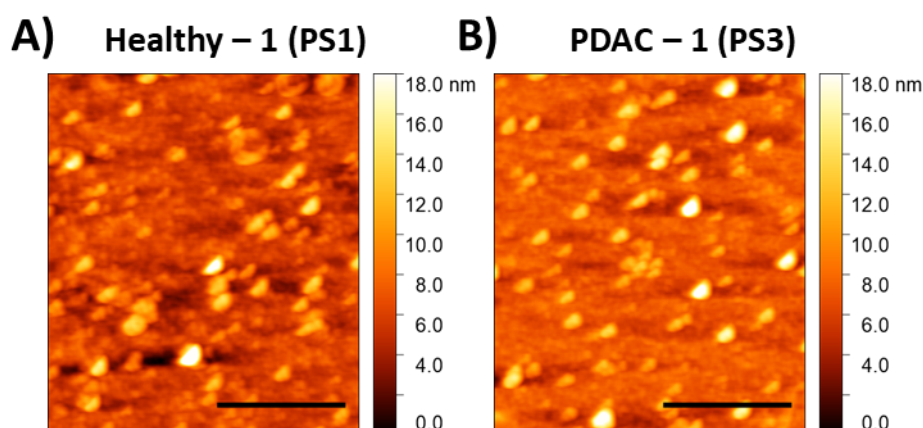


Figure 6-31 Atomic force microscopy images of functionalized graphene surface incubated with healthy plasma sample (PS1). G) AFM images of functionalized graphene surface incubated with PDAC plasma sample (PS3). AFM scale bars = 1 μ m.

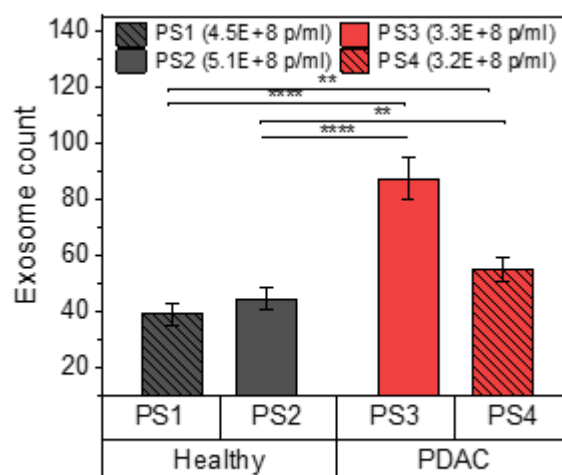


Figure 6-32 Exosome count on GFET sensors taken from an average of eight spots on each of the functionalized graphene surfaces incubated with healthy plasma samples (PS1 and PS2) and eight spots on incubated with PDAC plasma samples (PS3 and PS4). (**P < 0.01, ****P < 0.0001). Data are mean $\pm$ s.d.

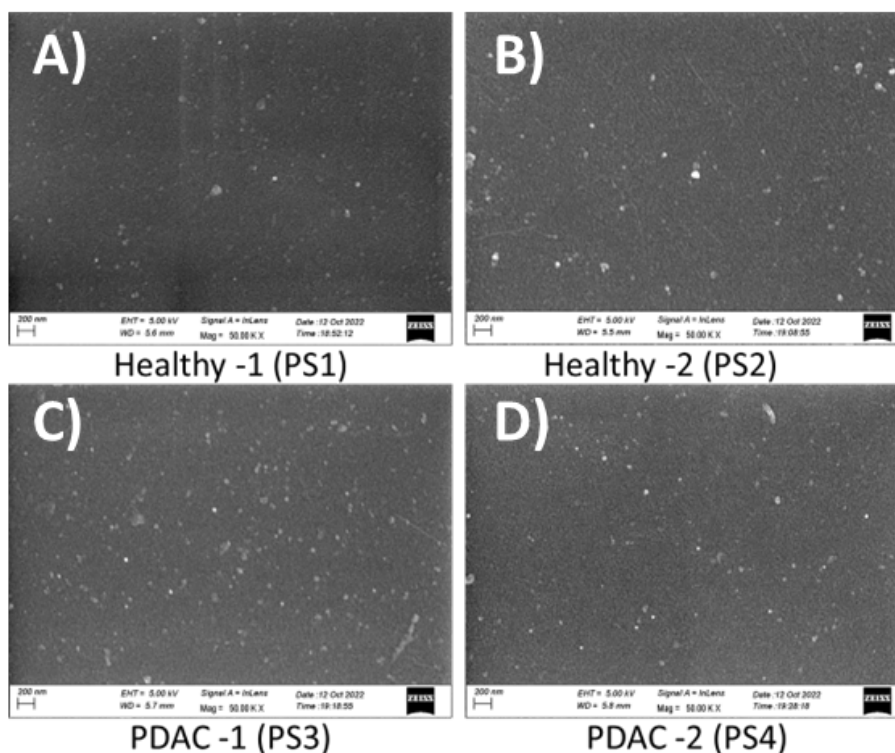


Figure 6-33 Comparison of exosomes captured on functionalized GFET sensors, scanning electron microscopy analysis of exosomes from healthy plasma samples: A) PS1 and B) PS2 and PDAC plasma samples. C) PS3 and D) PS4 captured on the functionalized graphene surface. SEM scale bars = 200 nm.

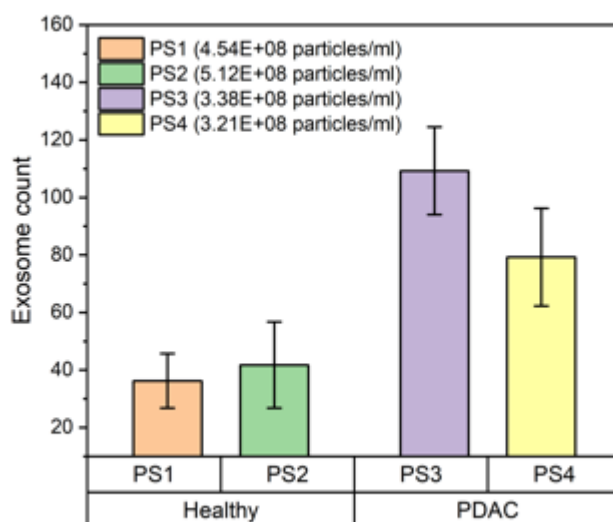


Figure 6-34 Exosome counts on graphene incubated with different samples, taken from an average of four spots on each graphene surface.

The results were found to be consistent with those from fluorescence imaging. Based on scanned areas of $800 \mu\text{m}^2$ for AFM and $100 \mu\text{m}^2$ for SEM for each sample, it was found that there was an average of 55.3% times and 58.6% times higher percentage of PDAC exosomes on the graphene surface compared with healthy exosomes. In the measurements using Nano Flow cytometry, the exosomes in healthy control and PDAC patient samples have similar size distributions ($80.0 \pm 5.8 \text{ nm}$ for PDAC exosomes; $85.7 \pm 6.7 \text{ nm}$ for healthy exosomes). Therefore, diffusion does not play a role in the enhancement of PDAC capture on the sensor surface. The higher response from the GFET sensor to PDAC samples could be due to higher GPC-1 expression on exosomes from PDAC plasma samples compared to those from healthy plasma samples,[287], [333] leading to a higher binding affinity between the antibody and PDAC exosomes. Therefore, exosomes from PDAC patient blood plasma and healthy control blood plasma were isolated.

6.8.3 Exosome isolation and characterisation of isolated exosomes from plasma samples

Several characterisation techniques were performed in order to ensure the isolated extracellular vesicles from the plasma are specifically exosome. Dynamic light scattering was performed, the size of particles lies between 30 and 130 nm, with an average diameter of 76.5 nm. (Figure 6-35) This size confirms the isolated extracellular vesicles are exosomes. The sizes of the isolated extracellular vesicles are between 30 – 100 nm were also shown in the immunogold TEM. The results from DLS and immunogold TEM confirmed the isolated extracellular vesicles from the plasma are specifically exosomes. The data from DLS in the supporting information. Prior to the analysis with NanoFCM to determine the concentrations of GPC-1+ exosomes in the plasma samples, samples were analyzed unstained first. It is shown from the NanoFCM analysis that the particles concentration are between 4.51×10^9 to 3.22×10^{11} particles/mL, with an average concentration of 5.85×10^{10} particles/mL.

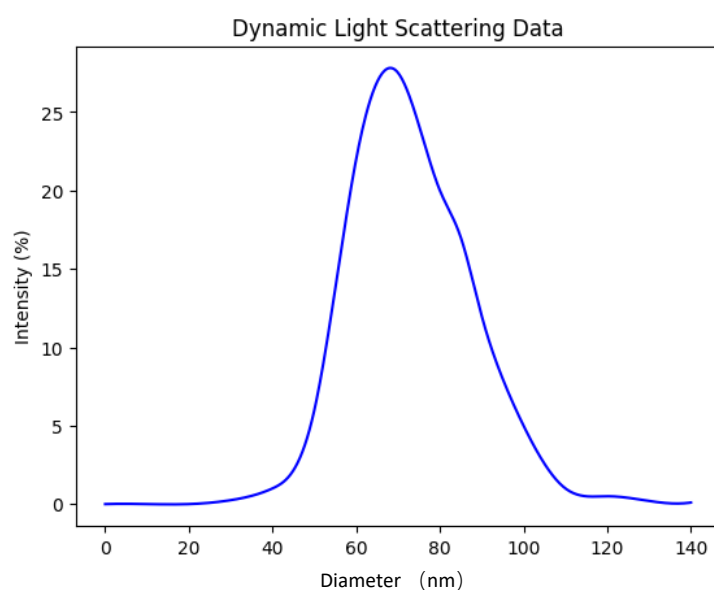


Figure 6-35 Dynamic light scattering measurements of isolated exosomes from blood plasma samples. The DLS showed an average size of 76.5 nm, with the size of particles lies between 30 and 130 nm.

Western blot analysis was performed to examine the isolation and expression of GPC-1 proteins on isolated plasma exosomes (Figure 6-36 and Figure 6-37). An elevated level of both CD9 and CD63 as exosomal markers[321], [334] was detected. (Figure 6-36) These results indicate that the exosomes were successfully isolated from patient and healthy control plasma samples.

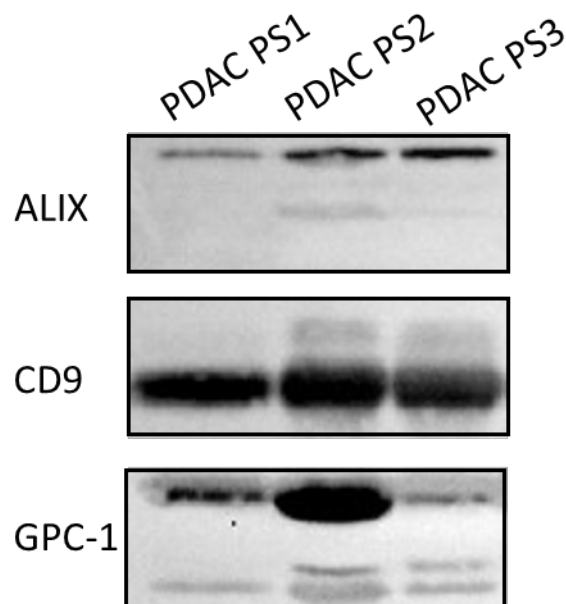


Figure 6-36 Western blot analyses of exosomes isolated from 3 PDAC patients' plasma, with ALIX antibody, CD9 antibody, and GPC-1 antibody.

Furthermore, the Western blot results suggest that the exosomal GPC-1 protein levels in the PDAC patient samples were significantly higher than those in healthy control samples. (Figure 6-37) This indicates a much higher GPC-1 protein expression on PDAC exosomes.

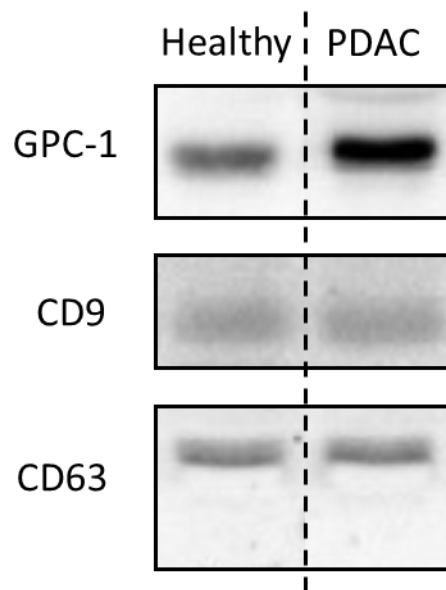


Figure 6-37 Western blot and IG-TEM validation of GPC-1 expression on PDAC cancerous and healthy exosomes. A) Western blot of GPC-1, CD9 and CD63 in exosomes extracted from healthy plasma and PDAC patient plasma.

To further validate the high expression of GPC-1 protein on PDAC exosome, TEM imaging with immunogold labelling was performed to directly reveal the GPC-1 proteins density on the surface of healthy and cancerous exosomes. TEM with immunogold labelling identified large number of GPC-1 proteins are exhibited on the surface of cancerous exosomes while very small number of GPC-1 proteins are presented on the surface of healthy exosomes (Figure 6-38 and Figure 6-39).

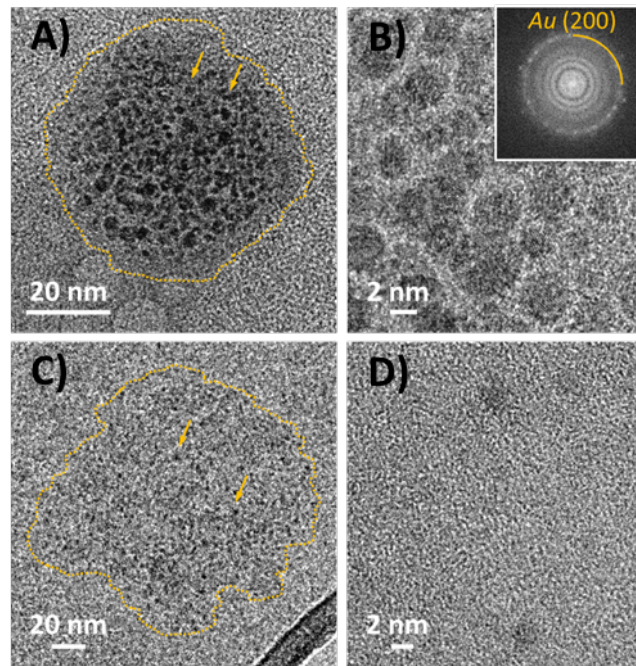


Figure 6-38 A) Low-magnification IG-TEM image of GPC-1 in PDAC cancer exosomes with immunogold labelling. The outlines of individual exosomes are highlighted by dashed yellow line for clarity. B) High-resolution IG-TEM image of GPC-1 on the surface of a PDAC cancer exosome with immunogold labelling. Inset shows the respective FFT pattern with the prominent ring corresponding to the (200) planes of Au. C) Low-magnification IG-TEM image of a healthy exosome with immunogold labelling. The yellow circles around individual exosomes are given for visual guidance. The arrows point to the individual Au particles on the surface of a healthy exosome. D) High-resolution IG-TEM image of GPC-1 on the surface of a healthy exosome with immunogold labelling.

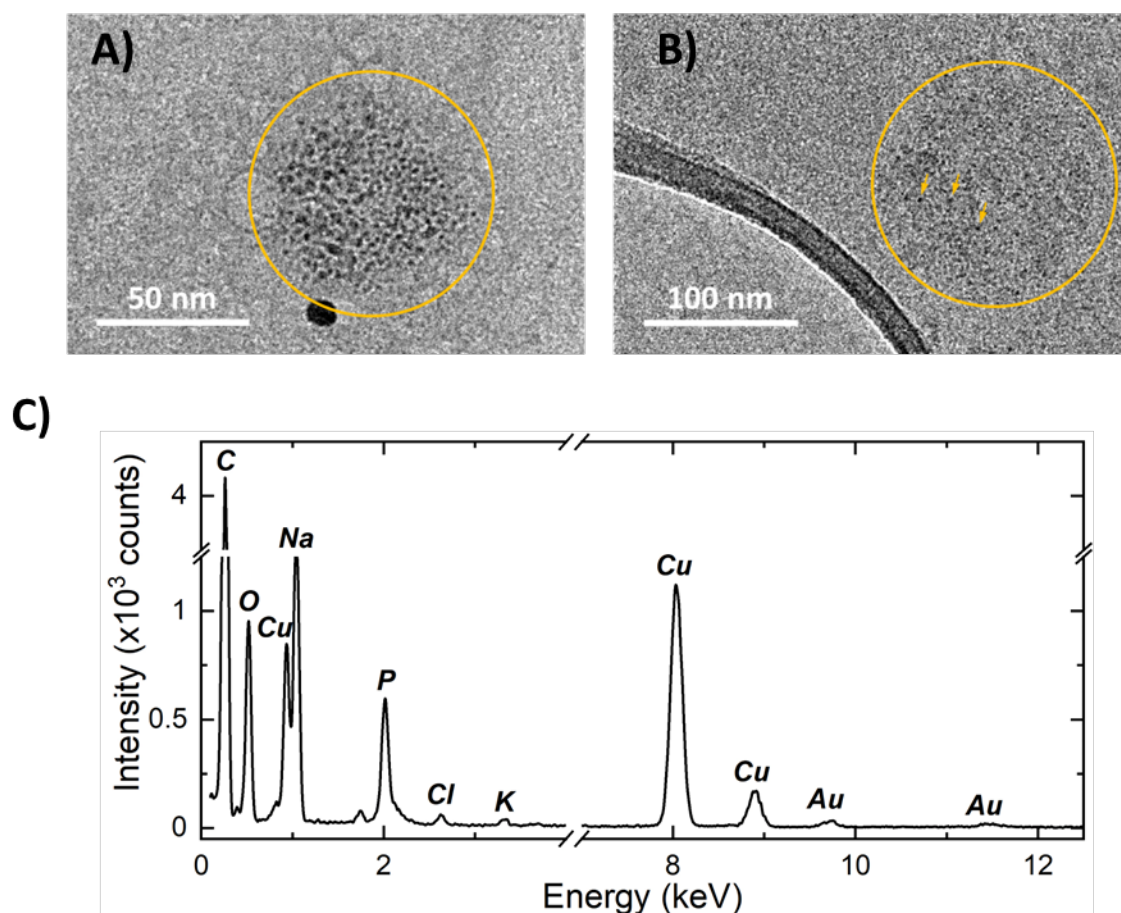


Figure 6-39 A) Low-magnification IG-TEM image of GPC-1 in PDAC cancer exosomes with immunogold labelling. The outlines of individual exosomes are highlighted by dashed yellow line for clarity. B) Low-magnification IG-TEM image of a healthy exosome with immunogold labelling. The yellow circles around individual exosomes are given for visual guidance. The arrows point to the individual Au particles on the surface of a healthy exosome. C) EDX spectrum of an Au-stained P-exosome showing the Au signal. The Cu signal originates from the TEM grid.

6.8.4 Discussion

The multiple GPC-1 proteins on the surface of the cancerous exosomes can increase the accessibility of the exosomes to the antibodies on the surface by increasing the number of proteins expressed on the exosome surface. Furthermore, the presence of multiple available sites on the exosome surface can promote cooperative reactions and increase the vividity and affinity to the antibody.[335] For example, having many specific proteins on the exosome's surface could allow several adjacent antibodies to bind more effectively to the exosome. This

could explain the higher density of cancerous exosomes on the graphene surface relative to the healthy ones and a higher response from the GFET sensor to samples from PDAC patients than healthy controls, especially given that the numbers of exosomes present in the plasma were the same for both samples.

It is worth to note that there are many free circulating GPC-1 protein in the blood plasma and may lead to small false positive signals of the GFET biosensor. It is reported that the concentration of GPC-1 protein in plasma is small compared to circulating exosomes, generally between 8.74 – 32.67 ng/mL.[336] Meanwhile, the GPC-1 protein concentration should be observed both in healthy controls and PDAC cancer plasma samples. In the previous work,[337] it was found that there is no significant difference between the level of circulating GPC-1 proteins in healthy control's and PDAC patients' plasma.[337] During the clinical detection, a small shift was observed in the device response from healthy samples, which is the sum of GPC-1 protein + GPC-1+ exosome. These indicate that the contribution of the GPC-1 protein is negligible in comparison to the GPC-1+ exosome.

6.9 Analytical modeling of GFETs response to GPC-1 exosome

Furthermore, the GFETs response as a function of exosome concentration in plasma is plotted (Figure 6-40). A significant dependence of GFETs response on the logarithmic concentration of exosome in the plasma was found (Pearson' correlation coefficient = 0.585; Sig. p=0.002).

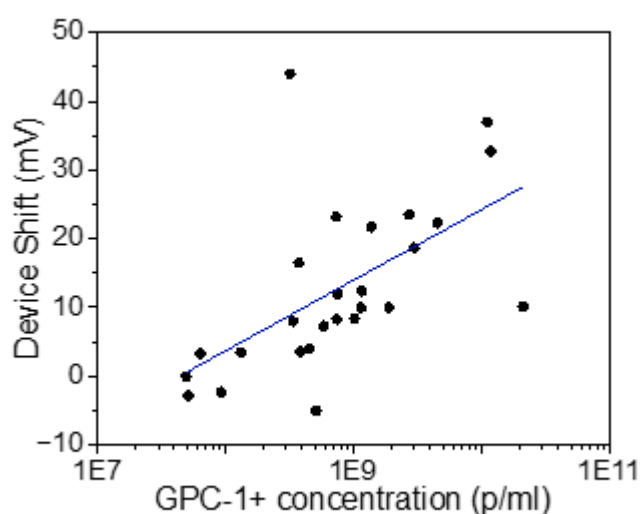


Figure 6-40 Analytical performance assessment of the GFET biosensor for GPC-1+ PDAC exosome detection in blood plasma samples: A) GFET biosensor device response against the GPC-1+ exosome concentration in blood plasma (Pearson correlation 0.585; Sig. 0.002).

Microscopy imaging of immobilized exosomes on the GFET sensor tested was further conducted to investigate the relationship between the GFET signal and the number of immobilized exosomes on the GFET sensing channel surface. Figure 6-41 shows AFM images of the exosomes on the GFET sensing channel after clinical testing.

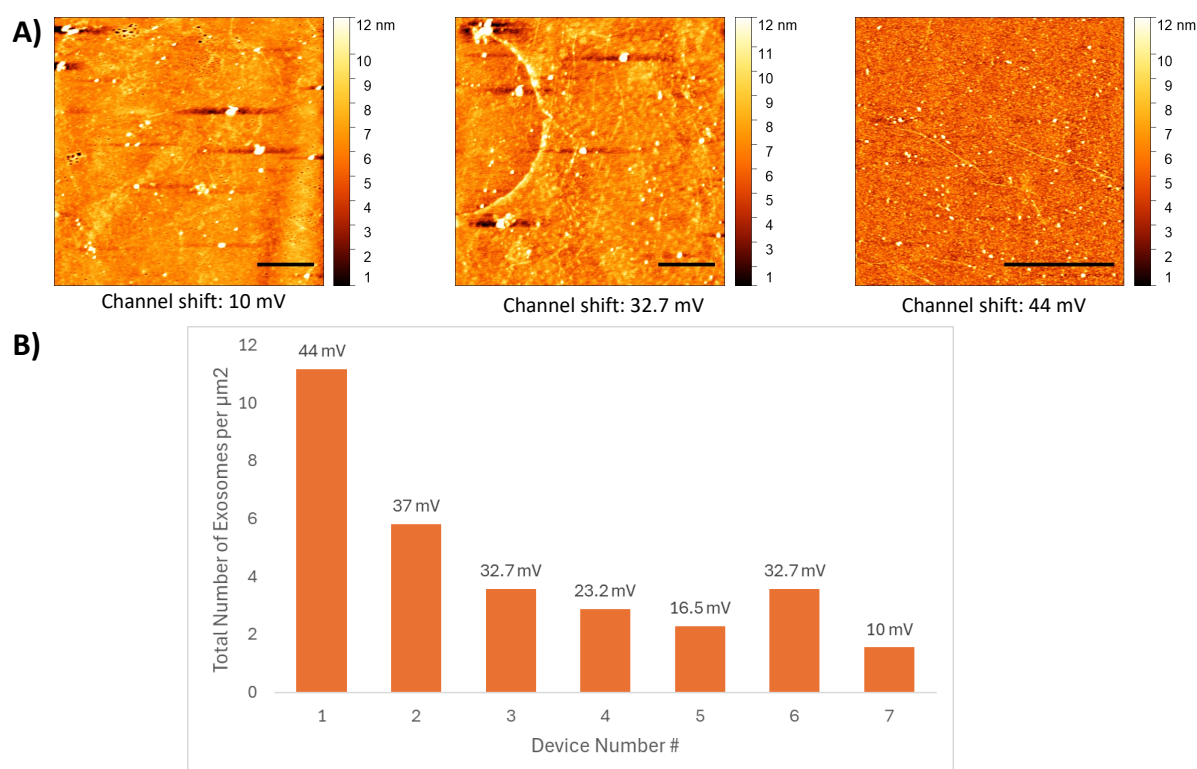


Figure 6-41 A) Atomic force microscopy images of exosome expression on surface of 3 different GFET biosensors with different device shift after clinical testing with 3 different PDAC patient plasma samples. AFM scale bars = 1 μm . B) Exosome counts from AFM on the GFET device after clinical testing, taken from an average of four spots on each graphene surface. The GFET device shift is labelled on the chart.

To explain the experimental observations of surface exosome concentration and GFET device response, an analytical model was established to look at the induced electrostatic surface potential on the graphene surface. This model is established based on the fixed exosomes on

the graphene surface and neglects the binding kinetics between the GFET sensor and exosomes. The total surface charge on the graphene surface can be written as:

$$\sigma_T = \sigma_G + \sigma_{DL} \quad (1)$$

where σ_T represents the net charge density of the exosomes on graphene surface, σ_G is the charge induced in graphene, and σ_{DL} is the net charge density in the electrical double layer. Based on the protocol for clinical testing, it was assumed that the exosomes immobilized on the graphene surface are fixed with fixed charges. The charges brought by exosomes binding to the graphene surface are assumed to be a uniform layer of charges. Equation 1 can be rewritten as:

$$\sigma_{exo} N_T = q n_G + \sigma_{DL} \quad (2)$$

where σ_{exo} is the charge of an exosome, N_T is the total number of exosomes on the graphene surface, n_G is the carrier density in graphene, and q is the electrical charge. As the carrier density is low at neutrality point, therefore the term $q n_G$ at the Dirac voltage can be neglected. The charge density in the electrical double layer can be obtained from the Poisson–Boltzmann equation and is given by:[338], [339]

$$\sigma_{DL} = \frac{2\epsilon_r \epsilon_0 k_d K T}{z q} \sinh\left(\frac{q \Psi_0}{2 K T}\right) \quad (3)$$

where k_d is:

$$k_d = \sqrt{\frac{2 q^2 N_{avo} I_0}{\epsilon_r \epsilon_0 K T}} \quad (4)$$

In Equation 4, ε_0 , ε_r , K , T , N_{avo} , and I_0 are the permittivity of the free space, the relative permittivity in the PBS, temperature, the Avogadro's constant, and the ion concentration respectively. Therefore:

$$\sigma_b N_{\text{tot}} = \frac{2\varepsilon_r \varepsilon_0 k_d K T}{ze} \sinh\left(\frac{e\Psi_0}{2KT}\right) \quad (5)$$

$$\sinh\left(\frac{q\Psi_0}{2KT}\right) = \frac{e^{\left(\frac{q\Psi_0}{2KT}\right)} + e^{\left(\frac{-q\Psi_0}{2KT}\right)}}{2} \approx \frac{e^{\left(\frac{q\Psi_0}{2KT}\right)}}{2} \quad (6)$$

$$\Psi_0 = \frac{2KT}{q} \sinh^{-1}\left(\frac{\sigma_b N_{\text{tot}} q z}{2\varepsilon_r \varepsilon_0 K T k_d}\right) \quad (7)$$

The charges of exosomes have been estimated to range from 50e to 1300e electron;[340] however, some studies have reported higher exosome charges. In the model, the exosome charge was assumed to be 1300e taking into consideration their relatively large size and the fact that cancerous exosomes tend to exhibit higher charges on their surfaces.[332] The Debye length in the chosen buffer condition is approximately 23 nm.[68] The number of exosomes per μm^2 on the graphene surface was varied and extracted the induced surface potential as a function of unit area of exosome concentration, as shown in Figure 6-42. The results for the Dirac voltage shift of the GFET devices and the surface exosome concentration of the corresponding device is fitted to this model. The response of the fabricated GFET follows the same trend as that of the analytical model. Furthermore, a logarithmic dependent response can be observed between exosome concentration and the induced surface potential with large concentrations of exosomes on the graphene surface.

The statistical quantification of these AFM profiles shows the relationship between the count of exosomes on the GFET sensing channel and the device signal in Figure 6-42. A logarithmic

dependence relationship can be found between the GFET signal and number of exosomes captured on the sensor surface.

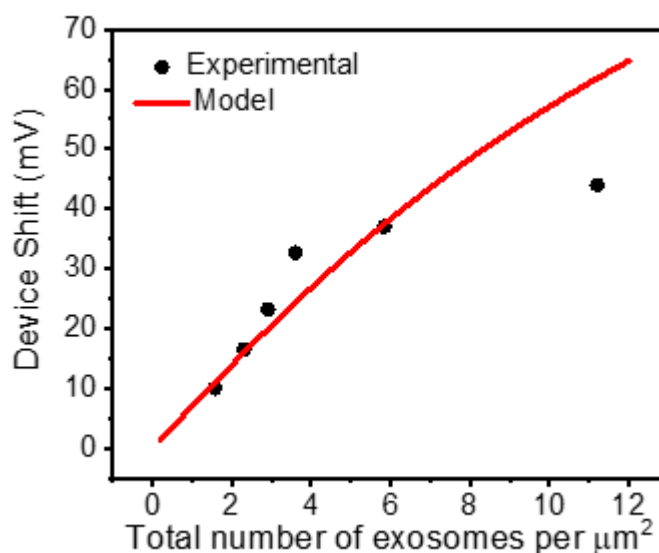


Figure 6-42 Correlation of detection signal from the GFET biosensor with the number of exosomes binding on the GFET sensing channel. The number of exosomes is counted based on average of four different spots of AFM scans of the sensing channel on the GFET biosensor. Error bars are determined by the standard deviation of exosome counts on graphene surface ($n = 4$).

6.10 Assessment of the GFET response versus the stage of cancer

Finally, the GFET signal with respect to the stage of PDAC cancer obtained from CT and MRI is summarized. The difference in the change of Dirac voltage of the GFET sensor as a function of cancer stage is plotted in Figure 6-43. Interestingly, the results indicate that the GFET biosensor detected all stages of PDAC cancer, including early-stage stage 1. However, the result shows no significant correlation between the signal of the GFET sensor and the stage of cancer. Moreover, there is no significant correlation between the concentration of exosomes in patient plasma and the stage of cancer ($P = 0.223$), which is consistent with previous findings in the literature. The independence of exosome concentration and PDAC stage indicates that the GFET biosensor technology could have high potential for use to detect PDAC cancer even at a very early stage of the condition.

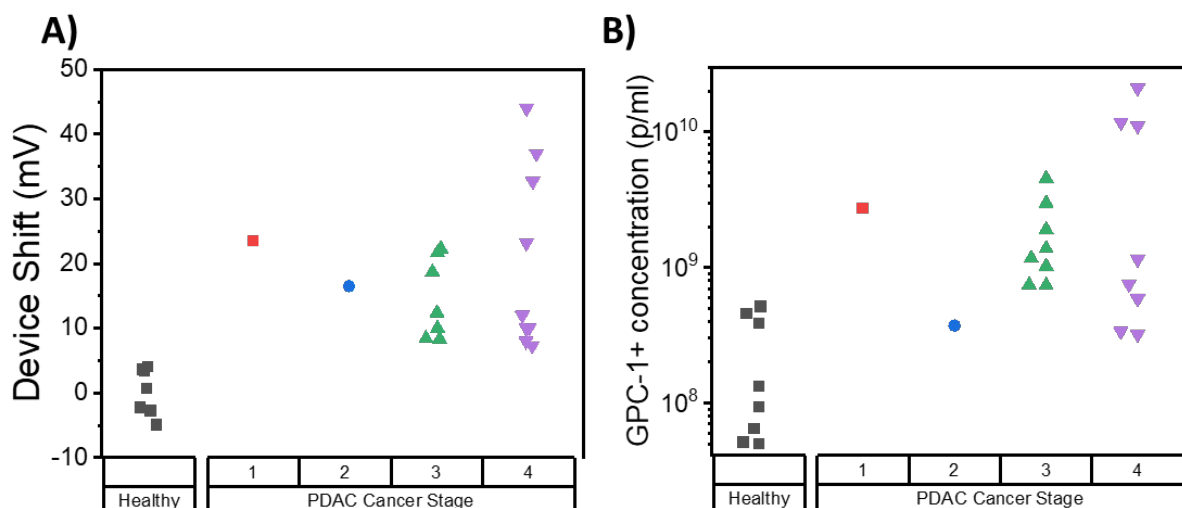


Figure 6-43 Detection of all PDAC cancer stages with the GFET biosensor. Measurement results of PDAC cancer stages, including stage 1, 2, 3, and 4, were classified using: A) Detection signals from the GFET biosensor; and B) GPC-1+ particle concentrations from Nano Flow cytometry measurements.

6.11 Conclusion

Pancreatic cancer (PC) is known for difficult early-stage detection and poor survival prognosis, and patients present for medical evaluation only when the cancer is advanced, and they experience signs and symptoms. Early detection can significantly improve the survival rate and outcome. In this study, an electrical test for the detection of cancerous exosomes using a GFET platform as a promising route for the early detection of pancreatic cancer is proposed. The test can be performed on whole plasma samples using a drop of 20 μ L with a total detection time of less than 45 min, which can be greatly reduced by using a faster readout electronic system. The test can detect all stages of cancer, taking advantage of the characteristics of cancer exosomes and the high sensitivity of GFETs. Based on samples from 26 patients, it is found that cancer exosomes such as GPC-1 exosomes are present in much higher levels in PDAC patient samples than in healthy ones. Although there was an overlap in the levels of exosomes in some samples, it is found that the density of exosomes on the GFET surface and the GFET response are significantly higher for cancer patient samples than those from healthy controls. After analysis of the GPC-1+ exosomes in pancreatic cancer and healthy

plasma using the Western blot analysis and IG-TEM, it is found that GPC-1 proteins are stronger expressed on pancreatic cancer exosomes compared to healthy ones. This could play a crucial role in the binding affinity and enables GPC-1 to have higher specificity to cancer exosomes than healthy ones, therefore increase the induced signal in the GFETs biosensors and improve the accuracy of detection. This high expression of charged proteins on the GPC-1+ cancer exosomes make GFETs sensors suitable for accurate discrimination between cancer and healthy samples which is otherwise challenging when other methods such as ELISA or Nanoflow are used particularly when the healthy and cancers plasma samples have the same level of GPC-1 exosome. The portable platform with read-in/out electronics makes the test simple and user-friendly, with no need for a trained professional to perform it. The presence of internal control on the GFET chip has significantly reduced false positive rates and improved the accuracy of detection. The test procedure consists of four simple steps including two blank measurements, incubation, and multiple rinsing which does not rely on skilled operator. The developed GFET platform is adaptable and can be used to detect multiple pancreatic cancer biomarkers simultaneously on the same chip. Beyond PDAC detection, this GFET technology can be reconfigured to facilitate the detection of other disease biomarkers which could be crucial for diagnosis purposes. Moreover, the GFET chip is CMOS compatible and can be manufactured on a large scale, which can massively reduce costs with small device-to-device variation and high yield.

7 Conclusions

Electrical biosensors based on graphene field-effect transistors (GFETs) have shown great potential for rapid and highly sensitive detection of a wide range of biomolecules. They are biocompatible, easy to functionalize, scalable for mass production, and can be incorporated into portable devices for point-of-care diagnostics. However, most of the reported GFET biosensors are performed with buffer and filtered biomarkers in lab conditions. The development of simple, sensitive, and scalable biosensors diagnosis tool that can detect with the physiological samples is significantly crucial to improve the outcome of highly lethal disease. However, real biological samples are often complexed in biological contents with high salinity, which introduce multiple challenges. The aim of this thesis is to develop of accurate, fast, and sensitive detection platform based on graphene field-effect-transistors (GFETs) for detection of disease biomarkers in physiological solutions. The performance of the GFETs biosensors are optimized in this thesis for achieving a uniform, sensitive, and selective GFET sensor with high device reliability and reproducibility.

Sensitivity and selectivity are of paramount importance for biomolecule detection in complex physiological solutions. The past decade has seen researchers develop and apply graphene technologies in biomarkers detection due to their advantages of real-time, label-free detection and high sensitivity. At the heart of these technologies is the immobilization of antibodies onto the graphene surface using noncovalent linker molecules. Yet, the critical role of these linker molecules in determining antibody orientation, and subsequently, the performance of graphene biosensors, has been largely uncharted. This crucial aspect in graphene biosensors is addressed by probing the orientation of antibodies on graphene surface functionalised using two different linker molecules, tetrakis (4-carboxyphenyl) porphyrin (TCPP) and 1-pyrenebutanoic acid succinimidyl ester (PBASE). Multiple characterisation techniques (XPS, Raman and AFM) were used first to characterize the presence of linker molecules and antibodies on the surface of graphene.

By probing the antibody orientation on the graphene surface using Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS), it is found that antibody adsorbed on TCPP-functionalized graphene exhibits dominant end-on vertical orientation, while mixed orientations of

antibodies are adsorbed on PBASE-functionalized graphene. Furthermore, Density-functional theory (DFT) simulations showed that the flexibility of the alkyl chain on the PBASE molecule can lead to different antibody orientations on graphene biosensors. Whereas the TCPP molecule stack on graphene has phenol rings bent up and tilted away from the surface of graphene, which leads to vertically orientated antibody. This study highlights the critical role of choosing the suitable linker molecule to achieve the proper antibody orientation on the graphene, which can profoundly influence the potential use of GFETs as ultra-sensitive biosensors for detecting disease biomarkers.

Following the optimization of the functionalization process of GFET biosensors with a new linker molecule, the GFET device was fabricated and integrated with a microfluidic channel on its surface for biosensing with liquid samples. A fundamental proof of principal study for investigating the performance of graphene functionalised using porphyrin linker molecules is demonstrated, both surface characterisation techniques and electrical measurements were performed. The experimental results from both detection in buffer and real clinical plasma samples were shown. The use of clinical samples and the results were also included to validate the sensor's capabilities. Using TCPP as a linker molecule on graphene, a two orders of magnitude enhancement in the limit of detection (LoD) of a GFET biosensor for exosome biomarker detection, compared to GFETs functionalized with PBASE is observed. Additionally, the graphene surface functionalized with the TCPP method showed a threefold increase in exosome density compared with PBASE-functionalized graphene. The effectiveness of the proposed functionalization structure is further demonstrated by employing GFETs to detect pancreatic cancer exosomes in patient plasma samples. The key findings of my study suggested that the use of porphyrin linker molecules can significantly enhance the sensing performance of graphene-based biosensors. This method can be adapted by all graphene-based biosensors in conjunction with bioreceptor molecules having amino groups. This technique is universal and gives the novelty of the GFET sensors and my PhD study.

Finally, the developed GFET is validated for biosensing applications with real physiological solutions. An accurate, fast, and sensitive detection platform with portable electronics based on graphene field-effect-transistors (GFETs) for direct detection of pancreatic ductal adenocarcinoma (PDAC) in patients' plasma samples were developed and demonstrated. In

this study, GFETs were used to detect specific exosomes in patients' plasma samples due to their promising role as cancer biomarkers for the early detection of PC. The target cell surface proteoglycan was selected to be GPC-1 because it is specifically enriched on cancer-cell derived exosomes and it is found to be in higher concentration levels in PC patients than healthy controls. It is first confirmed the high specificity of GPC-1 than CD63, Prom1, and RhoB proteins. Then, 281 GFETs were used to detect GPC-1+ exosomes in plasma from 18 PDAC patients and 8 healthy samples using a 20 μ L within 45 min. The GFET technology is clearly shown to be able to discriminate between samples from healthy and PDAC patients. Then the GFET results were compared with MRI and CT data and it was found that the GFET biosensor detected all stages of PDAC cancer, including early-stage grade 1.

Using Nanoflow cytometry it was confirmed that GPC-1+ exosomes are present in much higher levels in PDAC patient samples than in healthy ones. Although there was an overlap in the levels of exosomes in some samples, the GFETs can still significantly distinguish between the two samples. Interestingly, using Western blot, it was found this high response of GFETS is because cancerous exosomes are much higher expressed with GPC-1 proteins than healthy samples which can result in an increase in binding affinity between antibodies and cancerous exosomes. This was confirmed using Fluorescence microscopy, AFM, and SEM that show the density of GPC-1+ cancerous exosomes on graphene surface is between 53-58% higher than healthy ones even if both exosomes are present in the same level in plasma. After analysis of the GPC-1+ exosomes in pancreatic cancer and healthy plasma using the Western blot analysis and IG-TEM, it was found that GPC-1 proteins are stronger expressed on pancreatic cancer exosomes compared to healthy ones. These results are specifically important because they make electrical detection of GPC-1 is a unique detection platform to distinguish between PC and healthy ones when other technologies could produce a high level of false negative.

Overall, I developed and demonstrated a chip-based biosensor technology based on graphene field effect transistor (gFET) arrays as the proof-of-principle concept for the detection of biomarkers in physiological solutions with blood plasma samples.

8 Outlook

The work presented in this thesis indicates the success of the first proof-of-principle GFETs biosensor for the detection of biomarkers in blood plasma samples in a clinical trial set-up environment. This is a starting point for the investigation and application of GFETs biosensor platform for the detection and monitoring of disease. There are many aspects that the device can be optimized and improved for the development and further clinical verification of a simple, fast, cost-effective, highly accurate and sensitive cancer diagnosis platform, which enables the detection of multiple disease biomarkers using simple portable electronics.

First, the performance of the GFET biosensor can be further optimized in order to achieve direct sensing in real physiological solutions without the need for rinsing. Secondly, the layout of the GFETs sensor array can be further designed for the multiplex detection. Therefore, multiple disease biomarkers can be detected simultaneously in the same clinical samples using GFETs array for a more accurate disease diagnostic and increased the statistical value of the detected graphene response. It is also possible to enhance the surface functionalisation by developing high affinity aptamers to enable faster reaction time and lower detection limits in comparison to antibodies.

8.1 Performance enhancement for GFET sensing in physiological solutions

- 1) Fabrication of GFETs on hydrophobic substrate, aiming to reduce the Debye screening length of graphene.

There is evidence that a hydrophobic substrate could effectively increase the double layer capacitance of graphene and therefore potentially increase the Debye screening length of GFET biosensors.[341] The improvement in the Debye screening length of graphene is believed to be related to the surface energy of the underlying substrate of graphene. This finding could possibly offer an alternative method to overcome the limitation of Debye screening in sensing.

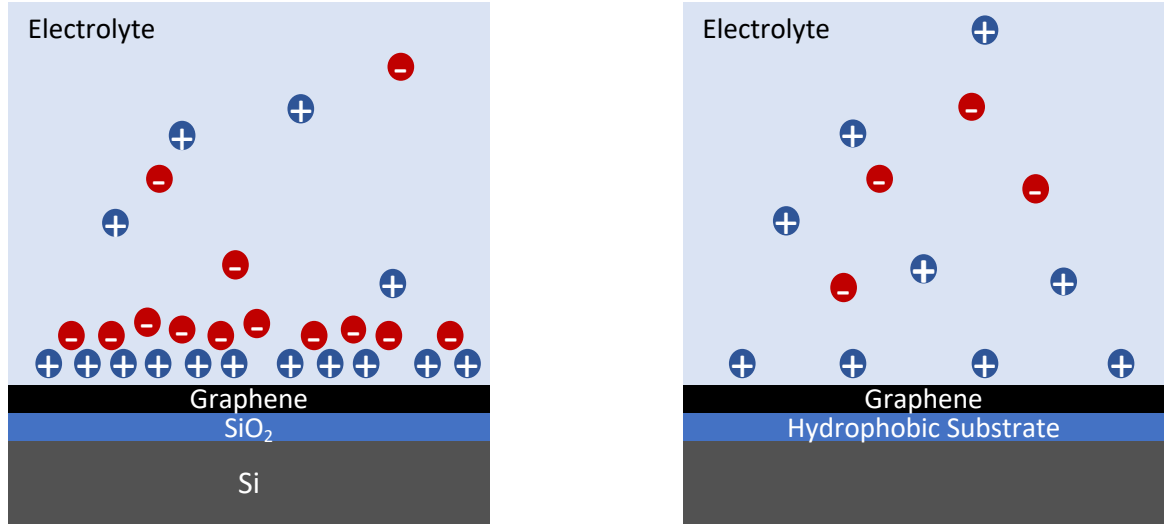


Figure 8-1 Schematic illustrations of the formation of electric double layer (EDL) on graphene with conventional Si/SiO₂ substrate and graphene with the hydrophobic substrate.

- 2) Nanostructuring of graphene surface to facilitate 3-dimensional diffusion of the target exosome towards the sensor surface, aiming to reduce incubation time.

The effect of nanostructuring on GFET biosensors are shown and discussed in details in Chapter 2.3.3.1. The application of nanostructuring on graphene biosensors can improve the accessibility of target bioanalytes to the surface of graphene, meanwhile it can enhance the diffusion of target bioanalytes to the surface of GFET. This enhancement in the diffusion can be explained by the diffusion equation:

$$N(t) = N_A C_0 t \left(\frac{A_D}{C_D(t)} + \frac{1}{K_{on} b_m} \right)^{-1}$$

where $N(t)$ is the number of targets that can be adsorbed on the surface of the GFET sensor at a given time, N_A is the Avogadro's number, C_0 is the initial target analyte concentration, $C_D(t)$ is the geometric diffusion capacitance. The diffusion equation indicates that the number of targets can be adsorbed on the surface of the GFET sensor is proportional to t for a GFET with spherical surface and proportional to $\sqrt{t}$ for a flat surface GFET. [342]

There are several different methods that can be employed for the fabrication of nanostructures on the surface of graphene. One of the approaches is to employ surface decoration with nanoparticles, such as carbon dots, quantum dots and metal nanoparticles.

-
- 3) Integration of Rayleigh wave streaming to enhance the mass transport of biomarkers to GFETs surface and reduce the non-specific bindings.

The detection of very low concentration of biomarkers are often limited by the binding kinetics of the target. For example, at a small sample volume of (100 μ L), where a single particle could present in the solution, the particle could take several hours to reach at the micro-nano sensor surface and stick without external force. Therefore, enhancing mass transport of the target to the sensor surface is critical to achieve single particle detection.

It has been recently reported by Huang et al. [343] that the sensitivity of shear horizontal surface acoustic wave (SH-SAW) biosensors can be improved through incorporating Rayleigh wave-based actuation. They achieved a fourfold improvement in sensitivity compared to conventional SH-SAW biosensors. The origin of the enhancement in the sensitivity is due to the acoustic streaming generated by the incident Rayleigh wave, which accelerate the mass transport of biomolecules in the solution to the sensor surface. This acoustic streaming formed can enhance the binding kinetics of the targets to the bioreceptor molecules on the surface, resulting in the improvement in the sensitivity and the limit of detection (LoD).

This application of the Rayleigh wave-based actuation can be applied and integrated on the GFET biosensors. This integration can generate a turbulence flow to the solution on top of the GFET sensor to enhance the mass transport of the targets to the sensor surface. Thus, achieving an improved sensitivity and LoD. Meanwhile, the application of the turbulence flow may also help in removing the non-specific binding events from the surface of the sensor, leading to better sensing performance.

8.2 Multiplexed detection using GFETs biosensors

Multiplexed detection is defined as the ability of the biosensor to simultaneously detect and analyze multiple biomolecules or target analytes in the same sample using the same chip. There is an increasing demand for multiplex detection platforms to detect biomarkers in body fluids. This is because analyzing a single biomarker lacks sufficient information for clinical

diagnosis. Furthermore, there is no single biomarker can be used for the diagnostic of disease. Simultaneous analysis of multiple biomarkers may provide a more reliable outcome, which may increase the accuracy of diagnosis. Meanwhile, minimal sample volume and preparation are required, with fast operation and turnaround detection time and lower cost per test.

The multiplexed detection using GFETs biosensors can be achieved with a GFET chip designed with several functional areas of GFET sensor arrays. Each functional area can be specifically functionalised with disease specific aptamer / antibody for the detection of target biomarkers in the solution. Each functional area will also consist of several GFETs channels to be used as a control. A house designed 3D printed moulds can be used to specific and selective incubation of antibodies on the different functional areas on the GFET chip.

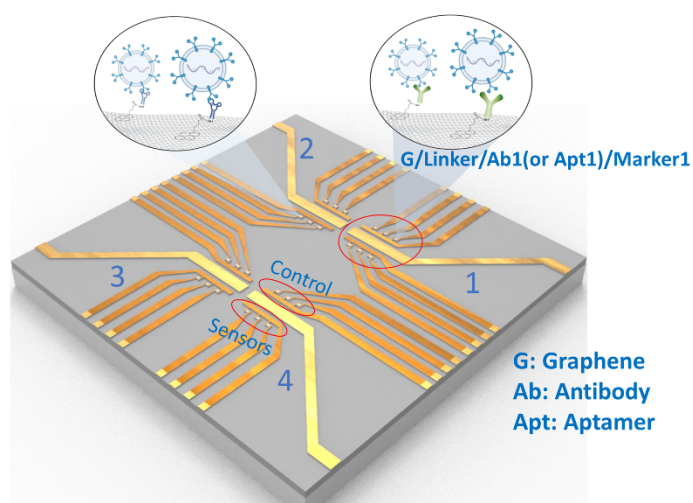


Figure 8-2 Schematics of the GFET chip for multiple biomarker detection. The chip compromises several functional areas of GFET sensor arrays. Each functional area contains the detection and negative controls for each biomarker.

References

- [1] S. Connal *et al.*, 'Liquid biopsies: the future of cancer early detection', *J. Transl. Med.*, vol. 21, no. 1, 2023, doi: 10.1186/s12967-023-03960-8.
- [2] P. Krzyszczyk *et al.*, 'The growing role of precision and personalized medicine for cancer treatment', *TECHNOLOGY*, vol. 06, no. 03n04, pp. 79–100, 2018, doi: 10.1142/s2339547818300020.
- [3] C. Wang, M. Liu, Z. Wang, S. Li, Y. Deng, and N. He, 'Point-of-care diagnostics for infectious diseases: From methods to devices', *Nano Today*, vol. 37, p. 101092, 2021, doi: 10.1016/j.nantod.2021.101092.
- [4] L. Xu, D. Li, S. Ramadan, Y. Li, and N. Klein, 'Facile biosensors for rapid detection of COVID-19', *Biosens. Bioelectron.*, vol. 170, p. 112673, Dec. 2020, doi: 10.1016/j.bios.2020.112673.
- [5] 'Deliver on diagnostics to save lives', *Nat. Microbiol.*, vol. 3, no. 8, pp. 847–847, 2018, doi: 10.1038/s41564-018-0220-9.
- [6] K. Clack, N. Soda, S. Kasetsirikul, R. G. Mahmudunnabi, N. Nguyen, and M. J. A. Shiddiky, 'Toward Personalized Nanomedicine: The Critical Evaluation of Micro and Nanodevices for Cancer Biomarker Analysis in Liquid Biopsy', *Small*, vol. 19, no. 15, p. 2205856, Apr. 2023, doi: 10.1002/smll.202205856.
- [7] R. Haldavnekar, K. Venkatakrishnan, and B. Tan, 'Cancer Stem Cell Derived Extracellular Vesicles with Self-Functionalized 3D Nanosensor for Real-Time Cancer Diagnosis: Eliminating the Roadblocks in Liquid Biopsy', *ACS Nano*, vol. 16, no. 8, pp. 12226–12243, Aug. 2022, doi: 10.1021/acsnano.2c02971.
- [8] Md. H. Haque *et al.*, 'Detection of regional DNA methylation using DNA-graphene affinity interactions', *Biosens. Bioelectron.*, vol. 87, pp. 615–621, Jan. 2017, doi: 10.1016/j.bios.2016.09.016.
- [9] B. J. Green, T. Saberi Safaei, A. Mephram, M. Labib, R. M. Mohamadi, and S. O. Kelley, 'Beyond the Capture of Circulating Tumor Cells: Next-Generation Devices and Materials', *Angew. Chem. Int. Ed.*, vol. 55, no. 4, pp. 1252–1265, Jan. 2016, doi: 10.1002/anie.201505100.
- [10] I.-H. Cho, D. H. Kim, and S. Park, 'Electrochemical biosensors: perspective on functional nanomaterials for on-site analysis', *Biomater. Res.*, vol. 24, no. 1, 2020, doi: 10.1186/s40824-019-0181-y.
- [11] H. Chen *et al.*, 'Fundamentals, Applications, and Future Directions of Bioelectrocatalysis', *Chem. Rev.*, vol. 120, no. 23, pp. 12903–12993, 2020, doi: 10.1021/acs.chemrev.0c00472.
- [12] J. Yoon, M. Shin, T. Lee, and J.-W. Choi, 'Highly Sensitive Biosensors Based on Biomolecules and Functional Nanomaterials Depending on the Types of Nanomaterials: A Perspective Review', *Materials*, vol. 13, no. 2, p. 299, 2020, doi: 10.3390/ma13020299.
- [13] D. Saini, 'Synthesis and functionalization of graphene and application in electrochemical biosensing', *Nanotechnol. Rev.*, vol. 5, no. 4, 2016, doi: 10.1515/ntrev-2015-0059.

-
- [14] V. Georgakilas *et al.*, 'Noncovalent Functionalization of Graphene and Graphene Oxide for Energy Materials, Biosensing, Catalytic, and Biomedical Applications', *Chem. Rev.*, vol. 116, no. 9, pp. 5464–5519, 2016, doi: 10.1021/acs.chemrev.5b00620.
- [15] K. S. Kim *et al.*, 'Large-scale pattern growth of graphene films for stretchable transparent electrodes', *Nature*, vol. 457, no. 7230, pp. 706–710, 2009, doi: 10.1038/nature07719.
- [16] X. Li *et al.*, 'Large-Area Synthesis of High-Quality and Uniform Graphene Films on Copper Foils', *Science*, vol. 324, no. 5932, pp. 1312–1314, 2009, doi: 10.1126/science.1171245.
- [17] L. Xu *et al.*, 'On-chip integrated graphene aptasensor with portable readout for fast and label-free COVID-19 detection in virus transport medium', *Sens. Diagn.*, 2022, doi: 10.1039/d2sd00076h.
- [18] D. Park *et al.*, 'Multiplexed femtomolar detection of Alzheimer's disease biomarkers in biofluids using a reduced graphene oxide field-effect transistor', *Biosens. Bioelectron.*, vol. 167, p. 112505, 2020, doi: 10.1016/j.bios.2020.112505.
- [19] Y. J. Hwang *et al.*, 'Multiplexed DNA-functionalized graphene sensor with artificial intelligence-based discrimination performance for analyzing chemical vapor compositions', *Microsyst. Nanoeng.*, vol. 9, no. 1, p. 28, Mar. 2023, doi: 10.1038/s41378-023-00499-y.
- [20] T. Yin *et al.*, 'Graphene Sensor Arrays for Rapid and Accurate Detection of Pancreatic Cancer Exosomes in Patients' Blood Plasma Samples', *ACS Nano*, Jul. 2023, doi: 10.1021/acsnano.3c01812.
- [21] A. K. Geim and K. S. Novoselov, 'THE RISE OF GRAPHENE', p. 14.
- [22] K. M. Janavika and R. Prakash Thangaraj, 'Graphene and its application: A review', *Mater. Today Proc.*, 2023, doi: 10.1016/j.matpr.2023.05.446.
- [23] A. R. Urade, I. Lahiri, and K. S. Suresh, 'Graphene Properties, Synthesis and Applications: A Review', *JOM*, vol. 75, no. 3, pp. 614–630, 2022, doi: 10.1007/s11837-022-05505-8.
- [24] Igor Ilyakov *et al.*, 'Ultrafast Tunable Terahertz-to-Visible Light Conversion through Thermal Radiation from Graphene Metamaterials', *Nano Lett.*, vol. 23, no. 9, pp. 3872–3878, 2023, doi: 10.1021/acs.nanolett.3c00507.
- [25] I. Domingos *et al.*, 'Printed graphene electrodes for textile-embedded triboelectric nanogenerators for biomechanical sensing', *Nano Energy*, vol. 115, p. 108688, 2023, doi: 10.1016/j.nanoen.2023.108688.
- [26] E. Torres Alonso, D. Shin, G. Rajan, A. I. S. Neves, S. Russo, and M. F. Craciun, 'Water-Based Solution Processing and Wafer-Scale Integration of All-Graphene Humidity Sensors', *Adv. Sci.*, p. 1802318, 2019, doi: 10.1002/advs.201802318.
- [27] P. Avouris, 'Graphene: Electronic and Photonic Properties and Devices', *Nano Lett.*, vol. 10, no. 11, pp. 4285–4294, 2010, doi: 10.1021/nl102824h.

-
- [28] T. Ando, 'The electronic properties of graphene and carbon nanotubes', *NPG Asia Mater.*, vol. 1, no. 1, pp. 17–21, 2009, doi: 10.1038/asiamat.2009.1.
- [29] Z. Yan *et al.*, 'Toward the Synthesis of Wafer-Scale Single-Crystal Graphene on Copper Foils', *ACS Nano*, vol. 6, no. 10, pp. 9110–9117, 2012, doi: 10.1021/nn303352k.
- [30] J. N. Coleman, 'Liquid Exfoliation of Defect-Free Graphene', *Acc. Chem. Res.*, vol. 46, no. 1, pp. 14–22, 2012, doi: 10.1021/ar300009f.
- [31] P. K. M. K., S. Shanthini, and C. Srivastava, 'Electrochemical exfoliation of graphite for producing graphene using saccharin', *RSC Adv.*, vol. 5, no. 66, pp. 53865–53869, 2015, doi: 10.1039/c5ra07846f.
- [32] Z. Yan, Z. Peng, and J. M. Tour, 'Chemical Vapor Deposition of Graphene Single Crystals', *Acc. Chem. Res.*, vol. 47, no. 4, pp. 1327–1337, Apr. 2014, doi: 10.1021/ar4003043.
- [33] W. Zhang, P. Wu, Z. Li, and J. Yang, 'First-Principles Thermodynamics of Graphene Growth on Cu Surfaces', *J. Phys. Chem. C*, vol. 115, no. 36, pp. 17782–17787, 2011, doi: 10.1021/jp2006827.
- [34] X. Zhang, L. Wang, J. Xin, B. I. Yakobson, and F. Ding, 'Role of Hydrogen in Graphene Chemical Vapor Deposition Growth on a Copper Surface', *J. Am. Chem. Soc.*, vol. 136, no. 8, pp. 3040–3047, 2014, doi: 10.1021/ja405499x.
- [35] A. J. Strudwick *et al.*, 'Chemical Vapor Deposition of High Quality Graphene Films from Carbon Dioxide Atmospheres', *ACS Nano*, vol. 9, no. 1, pp. 31–42, 2014, doi: 10.1021/nn504822m.
- [36] M. Saeed, Y. Alshammari, S. A. Majeed, and E. Al-Nasrallah, 'Chemical Vapour Deposition of Graphene—Synthesis, Characterisation, and Applications: A Review', *Molecules*, vol. 25, no. 17, p. 3856, 2020, doi: 10.3390/molecules25173856.
- [37] M. Esmailpour, P. Bügel, K. Fink, F. Studt, W. Wenzel, and M. Kozłowska, 'Multiscale Model of CVD Growth of Graphene on Cu(111) Surface', *Int. J. Mol. Sci.*, vol. 24, no. 10, p. 8563, 2023, doi: 10.3390/ijms24108563.
- [38] Y. Song, W. Zou, Q. Lu, L. Lin, and Z. Liu, 'Graphene Transfer: Paving the Road for Applications of Chemical Vapor Deposition Graphene', *Small*, vol. 17, no. 48, 2021, doi: 10.1002/smll.202007600.
- [39] M.-A. Yoon, C. Kim, J.-H. Kim, H.-J. Lee, and K.-S. Kim, 'Surface Properties of CVD-Grown Graphene Transferred by Wet and Dry Transfer Processes', *Sensors*, vol. 22, no. 10, p. 3944, 2022, doi: 10.3390/s22103944.
- [40] D.-Y. Wang *et al.*, 'Clean-Lifting Transfer of Large-area Residual-Free Graphene Films', *Adv. Mater.*, vol. 25, no. 32, pp. 4521–4526, 2013, doi: 10.1002/adma.201301152.
- [41] H. H. Kim, Y. Chung, E. Lee, S. K. Lee, and K. Cho, 'Water-Free Transfer Method for CVD-Grown Graphene and Its Application to Flexible Air-Stable Graphene Transistors', *Adv. Mater.*, vol. 26, no. 20, pp. 3213–3217, 2014, doi: 10.1002/adma.201305940.

-
- [42] J. Kang *et al.*, 'Efficient Transfer of Large-Area Graphene Films onto Rigid Substrates by Hot Pressing', *ACS Nano*, vol. 6, no. 6, pp. 5360–5365, 2012, doi: 10.1021/nn301207d.
- [43] N. Hong, D. Kireev, Q. Zhao, D. Chen, D. Akinwande, and W. Li, 'Roll-to-Roll Dry Transfer of Large-Scale Graphene', *Adv. Mater.*, vol. 34, no. 3, p. 2106615, 2021, doi: 10.1002/adma.202106615.
- [44] J. Seo *et al.*, 'Direct Graphene Transfer and Its Application to Transfer Printing Using Mechanically Controlled, Large Area Graphene/Copper Freestanding Layer', *Adv. Funct. Mater.*, vol. 28, no. 26, p. 1707102, 2018, doi: 10.1002/adfm.201707102.
- [45] S. Yang, S. Choi, F. Okello, K.-J. Kim, S. Choi, and C. Kim, 'All-Dry Transfer of Graphene Film by van der Waals Interactions', *Nano Lett.*, vol. 19, no. 6, pp. 3590–3596, 2019, doi: 10.1021/acs.nanolett.9b00555.
- [46] S. Ullah *et al.*, 'Graphene transfer methods: A review', *Nano Res.*, vol. 14, no. 11, pp. 3756–3772, 2021, doi: 10.1007/s12274-021-3345-8.
- [47] S. K. Krishnan, N. Nataraj, M. Meyyappan, and U. Pal, 'Graphene-Based Field-Effect Transistors in Biosensing and Neural Interfacing Applications: Recent Advances and Prospects', *Anal. Chem.*, vol. 95, no. 5, pp. 2590–2622, 2023, doi: 10.1021/acs.analchem.2c03399.
- [48] M. Bahri *et al.*, 'Toward clean and crackless polymer-assisted transfer of CVD-grown graphene and its recent advances in GFET-based biosensors', *Mater. Today Chem.*, vol. 22, pp. 100578–100578, 2021, doi: 10.1016/j.mtchem.2021.100578.
- [49] X. Zhang *et al.*, 'Ultrasensitive Field-Effect Biosensors Enabled by the Unique Electronic Properties of Graphene', *Small*, vol. 16, no. 15, p. 1902820, 2019, doi: 10.1002/smll.201902820.
- [50] A. Béraud, M. Sauvage, C. M. Bazán, M. Tie, A. Bencherif, and D. Bouilly, 'Graphene field-effect transistors as bioanalytical sensors: design, operation and performance', *The Analyst*, vol. 146, no. 2, pp. 403–428, 2021, doi: 10.1039/d0an01661f.
- [51] A. H. Castro Neto, F. Guinea, N. M. R. Peres, K. S. Novoselov, and A. K. Geim, 'The electronic properties of graphene', *Rev. Mod. Phys.*, vol. 81, no. 1, pp. 109–162, 2009, doi: 10.1103/revmodphys.81.109.
- [52] X. Zhang *et al.*, 'Ultrasensitive Field-Effect Biosensors Enabled by the Unique Electronic Properties of Graphene', *Small*, vol. 16, no. 15, p. 1902820, Apr. 2020, doi: 10.1002/smll.201902820.
- [53] A. Béraud, M. Sauvage, C. M. Bazán, M. Tie, A. Bencherif, and D. Bouilly, 'Graphene field-effect transistors as bioanalytical sensors: design, operation and performance', *The Analyst*, vol. 146, no. 2, pp. 403–428, 2021, doi: 10.1039/D0AN01661F.
- [54] P. Hess, 'Bonding, structure, and mechanical stability of 2D materials: the predictive power of the periodic table', *Nanoscale Horiz.*, vol. 6, no. 11, pp. 856–892, 2021, doi: 10.1039/d1nh00113b.

-
- [55] R. Steeno, M. C. Rodríguez González, S. Eyley, W. Thielemans, K. S. Mali, and S. De Feyter, 'Covalent Functionalization of Carbon Surfaces: Diaryliodonium versus Aryldiazonium Chemistry', *Chem. Mater.*, vol. 32, no. 12, pp. 5246–5255, Jun. 2020, doi: 10.1021/acs.chemmater.0c01393.
- [56] A. J. Clancy, H. Au, N. Rubio, G. O. Coulter, and M. S. P. Shaffer, 'Understanding and controlling the covalent functionalisation of graphene', *Dalton Trans.*, vol. 49, no. 30, pp. 10308–10318, 2020, doi: 10.1039/D0DT01589J.
- [57] J. Park and M. Yan, 'Covalent Functionalization of Graphene with Reactive Intermediates', *Acc. Chem. Res.*, vol. 46, no. 1, pp. 181–189, Jan. 2013, doi: 10.1021/ar300172h.
- [58] G. L. C. Paulus, Q. H. Wang, and M. S. Strano, 'Covalent Electron Transfer Chemistry of Graphene with Diazonium Salts', *Acc. Chem. Res.*, vol. 46, no. 1, pp. 160–170, Jan. 2013, doi: 10.1021/ar300119z.
- [59] C. Cao, Y. Zhang, C. Jiang, M. Qi, and G. Liu, 'Advances on Aryldiazonium Salt Chemistry Based Interfacial Fabrication for Sensing Applications', *ACS Appl. Mater. Interfaces*, vol. 9, no. 6, pp. 5031–5049, Feb. 2017, doi: 10.1021/acsami.6b16108.
- [60] V. Mishyn *et al.*, 'Controlled covalent functionalization of a graphene-channel of a field effect transistor as an ideal platform for (bio)sensing applications', *Nanoscale Horiz.*, vol. 6, no. 10, pp. 819–829, 2021, doi: 10.1039/D1NH00355K.
- [61] T. Rodrigues *et al.*, 'Highly performing graphene-based field effect transistor for the differentiation between mild-moderate-severe myocardial injury', *Nano Today*, vol. 43, p. 101391, Apr. 2022, doi: 10.1016/j.nantod.2022.101391.
- [62] R. Furlan de Oliveira, V. Montes-García, A. Ciesielski, and P. Samorì, 'Harnessing selectivity in chemical sensing via supramolecular interactions: from functionalization of nanomaterials to device applications', *Mater. Horiz.*, vol. 8, no. 10, pp. 2685–2708, 2021, doi: 10.1039/d1mh01117k.
- [63] V. Mishyn *et al.*, 'The holy grail of pyrene-based surface ligands on the sensitivity of graphene-based field effect transistors', *Sens. Diagn.*, p. 10.1039.D1SD00036E, 2022, doi: 10.1039/D1SD00036E.
- [64] X.-Y. Fan, R. Nouchi, L.-C. Yin, and K. Tanigaki, 'Effects of electron-transfer chemical modification on the electrical characteristics of graphene', *Nanotechnology*, vol. 21, no. 47, p. 475208, Nov. 2010, doi: 10.1088/0957-4484/21/47/475208.
- [65] C.-J. Shih *et al.*, 'Disorder Imposed Limits of Mono- and Bilayer Graphene Electronic Modification Using Covalent Chemistry', *Nano Lett.*, vol. 13, no. 2, pp. 809–817, Feb. 2013, doi: 10.1021/nl304632e.
- [66] J. Lu, A. Lipatov, N. S. Vorobeve, D. S. Muratov, and A. Sinitskii, 'Photoswitchable Monolayer and Bilayer Graphene Devices Enabled by In Situ Covalent Functionalization', *Adv. Electron. Mater.*, vol. 4, no. 8, p. 1800021, Aug. 2018, doi: 10.1002/aelm.201800021.

-
- [67] X. Dong *et al.*, 'The electrical properties of graphene modified by bromophenyl groups derived from a diazonium compound', *Carbon*, vol. 50, no. 4, pp. 1517–1522, Apr. 2012, doi: 10.1016/j.carbon.2011.11.029.
- [68] D. Kwong Hong Tsang *et al.*, 'Chemically Functionalised Graphene FET Biosensor for the Label-free Sensing of Exosomes', *Sci. Rep.*, vol. 9, no. 1, p. 13946, 2019, doi: 10.1038/s41598-019-50412-9.
- [69] I. A. Vacchi, C. Ménard-Moyon, and A. Bianco, 'Chemical Functionalization of Graphene Family Members', *Phys. Sci. Rev.*, vol. 2, no. 1, Jan. 2017, doi: 10.1515/psr-2016-0103.
- [70] A. Sinitskii, A. Dimiev, D. A. Corley, A. A. Fursina, D. V. Kosynkin, and J. M. Tour, 'Kinetics of Diazonium Functionalization of Chemically Converted Graphene Nanoribbons', *ACS Nano*, vol. 4, no. 4, pp. 1949–1954, Apr. 2010, doi: 10.1021/nn901899j.
- [71] P. Huang, H. Zhu, L. Jing, Y. Zhao, and X. Gao, 'Graphene Covalently Binding Aryl Groups: Conductivity Increases Rather than Decreases', *ACS Nano*, vol. 5, no. 10, pp. 7945–7949, Oct. 2011, doi: 10.1021/nn2023232.
- [72] E. Bekyarova, S. Sarkar, S. Niyogi, M. E. Itkis, and R. C. Haddon, 'Advances in the chemical modification of epitaxial graphene', *J. Phys. Appl. Phys.*, vol. 45, no. 15, p. 154009, Apr. 2012, doi: 10.1088/0022-3727/45/15/154009.
- [73] J. Greenwood *et al.*, 'Covalent Modification of Graphene and Graphite Using Diazonium Chemistry: Tunable Grafting and Nanomanipulation', *ACS Nano*, vol. 9, no. 5, pp. 5520–5535, May 2015, doi: 10.1021/acs.nano.5b01580.
- [74] V. Q. Nguyen *et al.*, 'Unprecedented Self-Organized Monolayer of a Ru(II) Complex by Diazonium Electroreduction', *J. Am. Chem. Soc.*, vol. 138, no. 30, pp. 9381–9384, Aug. 2016, doi: 10.1021/jacs.6b04827.
- [75] E. Bekyarova *et al.*, 'Chemical Modification of Epitaxial Graphene: Spontaneous Grafting of Aryl Groups', *J. Am. Chem. Soc.*, vol. 131, no. 4, pp. 1336–1337, Feb. 2009, doi: 10.1021/ja8057327.
- [76] D. W. Boukhvalov and M. I. Katsnelson, 'Tuning the gap in bilayer graphene using chemical functionalization: Density functional calculations', *Phys. Rev. B*, vol. 78, no. 8, p. 085413, Aug. 2008, doi: 10.1103/PhysRevB.78.085413.
- [77] N. N. Sergeeva *et al.*, 'A photochemical approach for a fast and self-limited covalent modification of surface supported graphene with photoactive dyes', *Nanotechnology*, vol. 29, no. 27, p. 275705, Jul. 2018, doi: 10.1088/1361-6528/aabf11.
- [78] S. Sarkar, E. Bekyarova, and R. C. Haddon, 'Covalent chemistry in graphene electronics', *Mater. Today*, vol. 15, no. 6, pp. 276–285, Jun. 2012, doi: 10.1016/S1369-7021(12)70118-9.
- [79] A. Kaplan *et al.*, 'Current and future directions in electron transfer chemistry of graphene', *Chem. Soc. Rev.*, vol. 46, no. 15, pp. 4530–4571, 2017, doi: 10.1039/C7CS00181A.
- [80] Z. Xia *et al.*, 'Electrochemical Functionalization of Graphene at the Nanoscale with Self-Assembling Diazonium Salts', *ACS Nano*, vol. 10, no. 7, pp. 7125–7134, 2016, doi: 10.1021/acs.nano.6b03278.

-
- [81] M. Jacquet *et al.*, 'Diazonium-Based Covalent Molecular Wiring of Single-Layer Graphene Leads to Enhanced Unidirectional Photocurrent Generation through the p-doping Effect', vol. 34, no. 8, pp. 3744–3758, 2022, doi: 10.1021/acs.chemmater.2c00088.
- [82] C. M. Bazán, Anouk Béraud, Minh Tho Nguyen, A. Bencherif, R. Martel, and D. Bouilly, 'Dynamic Gate Control of Aryldiazonium Chemistry on Graphene Field-Effect Transistors', vol. 22, no. 7, pp. 2635–2642, 2022, doi: 10.1021/acs.nanolett.1c04397.
- [83] G. E. Fenoy, W. A. Marmisollé, O. Azzaroni, and W. Knoll, 'Acetylcholine biosensor based on the electrochemical functionalization of graphene field-effect transistors', *Biosens. Bioelectron.*, vol. 148, p. 111796, Jan. 2020, doi: 10.1016/j.bios.2019.111796.
- [84] J. E. Kim *et al.*, 'Highly sensitive graphene biosensor by monomolecular self-assembly of receptors on graphene surface', *Appl. Phys. Lett.*, vol. 110, no. 20, p. 203702, May 2017, doi: 10.1063/1.4983084.
- [85] Z. Zhang, H. Huang, X. Yang, and L. Zang, 'Tailoring Electronic Properties of Graphene by π - π Stacking with Aromatic Molecules', *J. Phys. Chem. Lett.*, vol. 2, no. 22, pp. 2897–2905, Nov. 2011, doi: 10.1021/jz201273r.
- [86] S. Ramadan *et al.*, 'Carbon-Dot-Enhanced Graphene Field-Effect Transistors for Ultrasensitive Detection of Exosomes', *ACS Appl. Mater. Interfaces*, vol. 13, no. 7, pp. 7854–7864, 2021, doi: 10.1021/acsami.0c18293.
- [87] D. K. Ban *et al.*, 'A Single Multiomics Transistor for Electronic Detection of SARS-Cov2 Variants Antigen and Viral RNA Without Amplification', vol. 8, no. 11, 2023, doi: 10.1002/admt.202201945.
- [88] Z. Hao *et al.*, 'Modulating the Linker Immobilization Density on Aptameric Graphene Field Effect Transistors Using an Electric Field', vol. 5, no. 8, pp. 2503–2513, 2020, doi: 10.1021/acssensors.0c00752.
- [89] Dai Changhao *et al.*, 'Ultraprecise Antigen 10-in-1 Pool Testing by Multiantibodies Transistor Assay', vol. 143, no. 47, pp. 19794–19801, 2021, doi: 10.1021/jacs.1c08598.
- [90] H. Li *et al.*, 'Amplification-Free Detection of SARS-CoV-2 and Respiratory Syncytial Virus Using CRISPR Cas13a and Graphene Field-Effect Transistors', *Angew. Chem. Int. Ed.*, vol. 61, no. 32, 2022, doi: 10.1002/anie.202203826.
- [91] Z. Weng *et al.*, 'CRISPR-Cas12a Biosensor Array for Ultrasensitive Detection of Unamplified DNA with Single-Nucleotide Polymorphic Discrimination', vol. 8, no. 4, pp. 1489–1499, 2023, doi: 10.1021/acssensors.2c02495.
- [92] D. K. Ban *et al.*, 'Rapid self-test of unprocessed viruses of SARS-CoV-2 and its variants in saliva by portable wireless graphene biosensor', *Proc. Natl. Acad. Sci.*, vol. 119, no. 28, 2022, doi: 10.1073/pnas.2206521119.
- [93] G. Seo *et al.*, 'Rapid Detection of COVID-19 Causative Virus (SARS-CoV-2) in Human Nasopharyngeal Swab Specimens Using Field-Effect Transistor-Based Biosensor', *ACS Nano*, vol. 14, no. 4, pp. 5135–5142, 2020, doi: 10.1021/acsnano.0c02823.

-
- [94] X. Jin *et al.*, 'A field effect transistor modified with reduced graphene oxide for immunodetection of Ebola virus', vol. 186, no. 4, 2019, doi: 10.1007/s00604-019-3256-5.
- [95] D. Kim *et al.*, 'Detection of Alpha-Fetoprotein in Hepatocellular Carcinoma Patient Plasma with Graphene Field-Effect Transistor', *Sensors*, vol. 18, no. 11, p. 4032, 2018, doi: 10.3390/s18114032.
- [96] Y. Liu *et al.*, 'Giant enhancement in vertical conductivity of stacked CVD graphene sheets by self-assembled molecular layers', *Nat. Commun.*, vol. 5, no. 1, p. 5461, Dec. 2014, doi: 10.1038/ncomms6461.
- [97] S. Wang *et al.*, 'Graphene field-effect transistor biosensor for detection of biotin with ultrahigh sensitivity and specificity', *Biosens. Bioelectron.*, vol. 165, p. 112363, Oct. 2020, doi: 10.1016/j.bios.2020.112363.
- [98] J. A. Mann, T. Alava, H. G. Craighead, and W. R. Dichtel, 'Preservation of Antibody Selectivity on Graphene by Conjugation to a Tripod Monolayer', *Angew. Chem. Int. Ed.*, vol. 52, no. 11, pp. 3177–3180, 2013, doi: 10.1002/anie.201209149.
- [99] E. Piccinini *et al.*, 'Biofunctionalization of Graphene-Based FET Sensors through Heterobifunctional Nanoscaffolds: Technology Validation toward Rapid COVID-19 Diagnostics and Monitoring', *Adv. Mater. Interfaces*, vol. 9, no. 15, p. 2102526, 2022, doi: 10.1002/admi.202102526.
- [100] M. Cao *et al.*, 'Electrochemical and Theoretical Study of π - π Stacking Interactions between Graphitic Surfaces and Pyrene Derivatives', *J. Phys. Chem. C*, vol. 118, no. 5, pp. 2650–2659, 2014, doi: 10.1021/jp411979x.
- [101] J. A. Mann, J. Rodríguez-López, H. D. Abruña, and W. R. Dichtel, 'Multivalent Binding Motifs for the Noncovalent Functionalization of Graphene', *J. Am. Chem. Soc.*, vol. 133, no. 44, pp. 17614–17617, Nov. 2011, doi: 10.1021/ja208239v.
- [102] B. Hou and A. D. Radadia, 'Differential Stability of Biosensing Proteins on Transferred Mono/Bilayer Graphene', vol. 4, no. 2, pp. 675–681, 2018, doi: 10.1021/acsbmaterials.7b00379.
- [103] T. Kawata *et al.*, 'Improved sensitivity of a graphene FET biosensor using porphyrin linkers', *Jpn. J. Appl. Phys.*, vol. 57, no. 6, p. 065103, 2018, doi: 10.7567/jjap.57.065103.
- [104] R. Zhang and Y. Jia, 'A Disposable Printed Liquid Gate Graphene Field Effect Transistor for a Salivary Cortisol Test', *ACS Sens.*, vol. 6, no. 8, pp. 3024–3031, Aug. 2021, doi: 10.1021/acssensors.1c00949.
- [105] M. Garrido *et al.*, 'Supramolecular assembly of pyrene-tetrathiafulvalene hybrids on graphene: structure–property relationships and biosensing activity', *J. Mater. Chem. C*, vol. 9, no. 33, pp. 10944–10951, 2021, doi: 10.1039/D1TC01442K.
- [106] T. Terse-Thakoor, S. Badhulika, and A. Mulchandani, 'Graphene based biosensors for healthcare', *J. Mater. Res.*, vol. 32, no. 15, pp. 2905–2929, Aug. 2017, doi: 10.1557/jmr.2017.175.
- [107] L. Zhou *et al.*, 'Label-free graphene biosensor targeting cancer molecules based on non-covalent modification', *Biosens. Bioelectron.*, vol. 87, pp. 701–707, Jan. 2017, doi: 10.1016/j.bios.2016.09.025.

-
- [108] D.-J. Kim, I. Y. Sohn, J.-H. Jung, O. J. Yoon, N.-E. Lee, and J.-S. Park, 'Reduced graphene oxide field-effect transistor for label-free femtomolar protein detection', *Biosens. Bioelectron.*, vol. 41, pp. 621–626, Mar. 2013, doi: 10.1016/j.bios.2012.09.040.
- [109] B. Thakur *et al.*, 'Rapid detection of single E. coli bacteria using a graphene-based field-effect transistor device', *Biosens. Bioelectron.*, vol. 110, pp. 16–22, Jul. 2018, doi: 10.1016/j.bios.2018.03.014.
- [110] J. Chang *et al.*, 'Ultrasonic-assisted self-assembly of monolayer graphene oxide for rapid detection of Escherichia coli bacteria', *Nanoscale*, vol. 5, no. 9, p. 3620, 2013, doi: 10.1039/c3nr00141e.
- [111] F. Liu, Y. H. Kim, D. S. Cheon, and T. S. Seo, 'Micropatterned reduced graphene oxide based field-effect transistor for real-time virus detection', *Sens. Actuators B Chem.*, vol. 186, pp. 252–257, Sep. 2013, doi: 10.1016/j.snb.2013.05.097.
- [112] G. Seo *et al.*, 'Correction to Rapid Detection of COVID-19 Causative Virus (SARS-CoV-2) in Human Nasopharyngeal Swab Specimens Using Field-Effect Transistor-Based Biosensor', *ACS Nano*, vol. 14, no. 9, pp. 12257–12258, Sep. 2020, doi: 10.1021/acsnano.0c06726.
- [113] A. Roberts *et al.*, 'Graphene functionalized field-effect transistors for ultrasensitive detection of Japanese encephalitis and Avian influenza virus', *Sci. Rep.*, vol. 10, no. 1, p. 14546, Dec. 2020, doi: 10.1038/s41598-020-71591-w.
- [114] D. Kwong Hong Tsang *et al.*, 'Chemically Functionalised Graphene FET Biosensor for the Label-free Sensing of Exosomes', *Sci. Rep.*, vol. 9, no. 1, p. 13946, Dec. 2019, doi: 10.1038/s41598-019-50412-9.
- [115] S. Ramadan *et al.*, 'Carbon-Dot-Enhanced Graphene Field-Effect Transistors for Ultrasensitive Detection of Exosomes', *ACS Appl. Mater. Interfaces*, vol. 13, no. 7, pp. 7854–7864, Feb. 2021, doi: 10.1021/acsaami.0c18293.
- [116] K. Islam, A. Suhail, and G. Pan, 'A Label-Free and Ultrasensitive Immunosensor for Detection of Human Chorionic Gonadotrophin Based on Graphene FETs', *Biosensors*, vol. 7, no. 3, p. 27, Jul. 2017, doi: 10.3390/bios7030027.
- [117] W. Saleem, C. Salinas, B. Watkins, G. Garvey, A. C. Sharma, and R. Ghosh, 'Antibody functionalized graphene biosensor for label-free electrochemical immunosensing of fibrinogen, an indicator of trauma induced coagulopathy', *Biosens. Bioelectron.*, vol. 86, pp. 522–529, Dec. 2016, doi: 10.1016/j.bios.2016.07.009.
- [118] L. Xu *et al.*, 'Detection of Glial Fibrillary Acidic Protein in Patient Plasma Using On-Chip Graphene Field-Effect Biosensors, in Comparison with ELISA and Single-Molecule Array', *ACS Sens.*, vol. 7, no. 1, pp. 253–262, Jan. 2022, doi: 10.1021/acssensors.1c02232.
- [119] S. Wang, Z. Hossain, Y. Zhao, and T. Han, 'Graphene Field-Effect Transistor Biosensor', in *Graphene Field-Effect Transistor Biosensors*, Singapore: Springer Singapore, 2021, pp. 45–67. doi: 10.1007/978-981-16-1212-1_4.

-
- [120] C. Wang *et al.*, 'A label-free and portable graphene FET aptasensor for children blood lead detection', *Sci. Rep.*, vol. 6, no. 1, 2016, doi: 10.1038/srep21711.
- [121] L. Xu, R. Chopdat, D. Li, and K. T. Al-Jamal, 'Development of a simple, sensitive and selective colorimetric aptasensor for the detection of cancer-derived exosomes', *Biosens. Bioelectron.*, vol. 169, p. 112576, Dec. 2020, doi: 10.1016/j.bios.2020.112576.
- [122] L. Xu, R. Wang, L. C. Kelso, Y. Ying, and Y. Li, 'A target-responsive and size-dependent hydrogel aptasensor embedded with QD fluorescent reporters for rapid detection of avian influenza virus H5N1', *Sens. Actuators B Chem.*, vol. 234, pp. 98–108, Oct. 2016, doi: 10.1016/j.snb.2016.04.156.
- [123] R. Wang, L. Xu, and Y. Li, 'Bio-nanogate controlled enzymatic reaction for virus sensing', *Biosens. Bioelectron.*, vol. 67, pp. 400–407, May 2015, doi: 10.1016/j.bios.2014.08.071.
- [124] N. Nakatsuka *et al.*, 'Aptamer–field-effect transistors overcome Debye length limitations for small-molecule sensing', *Science*, vol. 362, no. 6412, pp. 319–324, Oct. 2018, doi: 10.1126/science.aao6750.
- [125] Z. Hao *et al.*, 'An Intelligent Graphene-Based Biosensing Device for Cytokine Storm Syndrome Biomarkers Detection in Human Biofluids', vol. 17, no. 29, pp. 2101508–2101508, 2021, doi: 10.1002/sml.202101508.
- [126] J. Upan *et al.*, 'A simple label-free electrochemical sensor for sensitive detection of alpha-fetoprotein based on specific aptamer immobilized platinum nanoparticles/carboxylated-graphene oxide', *Sci. Rep.*, vol. 11, no. 1, p. 13969, Dec. 2021, doi: 10.1038/s41598-021-93399-y.
- [127] S. Yang, F. Zhang, Z. Wang, and Q. Liang, 'A graphene oxide-based label-free electrochemical aptasensor for the detection of alpha-fetoprotein', *Biosens. Bioelectron.*, vol. 112, pp. 186–192, Jul. 2018, doi: 10.1016/j.bios.2018.04.026.
- [128] T. Ono, Y. Kanai, Y. Ohno, K. Maehashi, K. Inoue, and K. Matsumoto, 'Electrical Biosensor Using Graphene Field-Effect Transistor and Small Receptor Molecules', in *Carbon Related Materials: Commemoration for Nobel Laureate Professor Suzuki Special Symposium at IUMRS-ICAM2017*, S. Kaneko, M. Aono, A. Pruna, M. Can, P. Mele, M. Ertugrul, and T. Endo, Eds., Singapore: Springer Singapore, 2021, pp. 91–101. doi: 10.1007/978-981-15-7610-2_5.
- [129] T. Ozer, B. J. Geiss, and C. S. Henry, 'Review—Chemical and Biological Sensors for Viral Detection', *J. Electrochem. Soc.*, vol. 167, no. 3, p. 037523, Jan. 2020, doi: 10.1149/2.0232003JES.
- [130] N. Kikuchi and D. M. Kolpashchikov, 'A universal split spinach aptamer (USSA) for nucleic acid analysis and DNA computation', *Chem. Commun.*, vol. 53, no. 36, pp. 4977–4980, 2017, doi: 10.1039/C7CC01540B.
- [131] T. Li, S. Dong, and E. Wang, 'A Lead(II)-Driven DNA Molecular Device for Turn-On Fluorescence Detection of Lead(II) Ion with High Selectivity and Sensitivity', *J. Am. Chem. Soc.*, vol. 132, no. 38, pp. 13156–13157, Sep. 2010, doi: 10.1021/ja105849m.

-
- [132] K. Horii *et al.*, 'Development of a Sphingosylphosphorylcholine Detection System Using RNA Aptamers', *Molecules*, vol. 15, no. 8, pp. 5742–5755, Aug. 2010, doi: 10.3390/molecules15085742.
- [133] A. D. Keefe, S. Pai, and A. Ellington, 'Aptamers as therapeutics', *Nat. Rev. Drug Discov.*, vol. 9, no. 7, pp. 537–550, Jul. 2010, doi: 10.1038/nrd3141.
- [134] X. Wang *et al.*, 'A graphene aptasensor for biomarker detection in human serum', *Electrochimica Acta*, vol. 290, pp. 356–363, Nov. 2018, doi: 10.1016/j.electacta.2018.08.062.
- [135] H. Yoo, H. Jo, and S. S. Oh, 'Detection and beyond: challenges and advances in aptamer-based biosensors', *Mater. Adv.*, vol. 1, no. 8, pp. 2663–2687, 2020, doi: 10.1039/D0MA00639D.
- [136] D. C. Grahame, 'The Electrical Double Layer and the Theory of Electrocapillarity.', *Chem. Rev.*, vol. 41, no. 3, pp. 441–501, Dec. 1947, doi: 10.1021/cr60130a002.
- [137] N. Gao *et al.*, 'Specific detection of biomolecules in physiological solutions using graphene transistor biosensors', *Proc. Natl. Acad. Sci.*, vol. 113, no. 51, pp. 14633–14638, Dec. 2016, doi: 10.1073/pnas.1625010114.
- [138] S. Wei, X. Zou, J. Tian, H. Huang, W. Guo, and Z. Chen, 'Control of Protein Conformation and Orientation on Graphene', *J. Am. Chem. Soc.*, vol. 141, no. 51, pp. 20335–20343, Dec. 2019, doi: 10.1021/jacs.9b10705.
- [139] X. Zou *et al.*, 'Molecular Interactions between Graphene and Biological Molecules', *J. Am. Chem. Soc.*, vol. 139, no. 5, pp. 1928–1936, Feb. 2017, doi: 10.1021/jacs.6b11226.
- [140] T. Alava *et al.*, 'Control of the Graphene–Protein Interface Is Required To Preserve Adsorbed Protein Function', *Anal. Chem.*, vol. 85, no. 5, pp. 2754–2759, Mar. 2013, doi: 10.1021/ac303268z.
- [141] S. Kumar and S. H. Parekh, 'Linking graphene-based material physicochemical properties with molecular adsorption, structure and cell fate', *Commun. Chem.*, vol. 3, no. 1, p. 8, Dec. 2020, doi: 10.1038/s42004-019-0254-9.
- [142] T. Wu *et al.*, 'Fast growth of inch-sized single-crystalline graphene from a controlled single nucleus on Cu–Ni alloys', *Nat. Mater.*, vol. 15, no. 1, pp. 43–47, Jan. 2016, doi: 10.1038/nmat4477.
- [143] S. Ramadan *et al.*, 'Enhancing Structural Properties and Performance of Graphene-Based Devices Using Self-Assembled HMDS Monolayers', *ACS Omega*, vol. 6, no. 7, pp. 4767–4775, Feb. 2021, doi: 10.1021/acsomega.0c05631.
- [144] D. Welch, S. Shah, S. Ozev, and J. Blain Christen, 'Experimental and Simulated Cycling of ISFET Electric Fields for Drift Reset', *IEEE Electron Device Lett.*, vol. 34, no. 3, pp. 456–458, 2013, doi: 10.1109/led.2013.2240648.
- [145] N. Miyakawa *et al.*, 'Drift Suppression of Solution-Gated Graphene Field-Effect Transistors by Cation Doping for Sensing Platforms', *Sensors*, vol. 21, no. 22, p. 7455, 2021, doi: 10.3390/s21227455.

-
- [146] K. Mensah *et al.*, 'DNA Hybridization Measured with Graphene Transistor Arrays', vol. 9, no. 16, pp. 2000260–2000260, 2020, doi: 10.1002/adhm.202000260.
- [147] L. Soleymani, Z. Fang, E. H. Sargent, and S. O. Kelley, 'Programming the detection limits of biosensors through controlled nanostructuring', *Nat. Nanotechnol.*, vol. 4, no. 12, pp. 844–848, Dec. 2009, doi: 10.1038/nnano.2009.276.
- [148] M. T. Hwang *et al.*, 'Ultrasensitive detection of nucleic acids using deformed graphene channel field effect biosensors', *Nat. Commun.*, vol. 11, no. 1, 2020, doi: 10.1038/s41467-020-15330-9.
- [149] L. Soleymani *et al.*, 'Hierarchical Nanotextured Microelectrodes Overcome the Molecular Transport Barrier To Achieve Rapid, Direct Bacterial Detection', *ACS Nano*, vol. 5, no. 4, pp. 3360–3366, Apr. 2011, doi: 10.1021/nn200586s.
- [150] Z. Gao *et al.*, 'Scalable Production of Sensor Arrays Based on High-Mobility Hybrid Graphene Field Effect Transistors', *ACS Appl. Mater. Interfaces*, vol. 8, no. 41, pp. 27546–27552, Oct. 2016, doi: 10.1021/acsami.6b09238.
- [151] B.-Y. Kim *et al.*, 'Ultrarapid and ultrasensitive electrical detection of proteins in a three-dimensional biosensor with high capture efficiency', *Nanoscale*, vol. 7, no. 21, pp. 9844–9851, 2015, doi: 10.1039/C5NR00909J.
- [152] M. T. Hwang *et al.*, 'Ultrasensitive Detection of Dopamine, IL-6 and SARS-CoV-2 Proteins on Crumpled Graphene FET Biosensor', *Adv. Mater. Technol.*, vol. 6, no. 11, p. 2100712, 2021, doi: 10.1002/admt.202100712.
- [153] B. Cai, L. Huang, H. Zhang, Z. Sun, Z. Zhang, and G.-J. Zhang, 'Gold nanoparticles-decorated graphene field-effect transistor biosensor for femtomolar MicroRNA detection', *Biosens. Bioelectron.*, vol. 74, pp. 329–334, 2015, doi: 10.1016/j.bios.2015.06.068.
- [154] D. Dong, J. Zhang, R. Zhang, F. Li, Y. Li, and Y. Jia, 'Multiprobe Assay for Clinical SEPT9 Methylation Based on the Carbon Dot-Modified Liquid-Exfoliated Graphene Field Effect Transistor with a Potential to Present a Methylation Panorama', *ACS Omega*, vol. 5, no. 26, pp. 16228–16237, 2020, doi: 10.1021/acsomega.0c02022.
- [155] I. Park *et al.*, 'Detection of SARS-CoV-2 Virus Amplification Using a Crumpled Graphene Field-Effect Transistor Biosensor', *ACS Sens.*, vol. 6, no. 12, pp. 4461–4470, 2021, doi: 10.1021/acssensors.1c01937.
- [156] M. S. Mannoer, T. James, D. V. Ivanov, L. Beadling, and W. Braunlin, 'Nanogap Dielectric Spectroscopy for Aptamer-Based Protein Detection', *Biophys. J.*, vol. 98, no. 4, pp. 724–732, Feb. 2010, doi: 10.1016/j.bpj.2009.10.042.
- [157] Z. Ghobaei Namhil *et al.*, 'A label-free aptamer-based nanogap capacitive biosensor with greatly diminished electrode polarization effects', *Phys. Chem. Chem. Phys.*, vol. 21, no. 2, pp. 681–691, 2019, doi: 10.1039/C8CP05510F.

-
- [158] K. Shoorideh and C. O. Chui, 'On the origin of enhanced sensitivity in nanoscale FET-based biosensors', *Proc. Natl. Acad. Sci.*, vol. 111, no. 14, pp. 5111–5116, Apr. 2014, doi: 10.1073/pnas.1315485111.
- [159] B. Li, Y. Zhang, X.-Y. Meng, and R. Zhou, 'Zipper-Like Unfolding of dsDNA Caused by Graphene Wrinkles', *J. Phys. Chem. C*, vol. 124, no. 5, pp. 3332–3340, Feb. 2020, doi: 10.1021/acs.jpcc.9b08778.
- [160] Y. Li, B. Li, Y. Liu, Y. Qu, J. Tian, and W.-J. Li, 'A wrinkled nanosurface causes accelerated protein unfolding revealing its critical role in nanotoxicity', vol. 12, no. 48, pp. 30976–30984, 2022, doi: 10.1039/d2ra05489b.
- [161] N. Gao, W. Zhou, X. Jiang, G. Hong, T.-M. Fu, and C. M. Lieber, 'General Strategy for Biodetection in High Ionic Strength Solutions Using Transistor-Based Nanoelectronic Sensors', *Nano Lett.*, vol. 15, no. 3, pp. 2143–2148, Mar. 2015, doi: 10.1021/acs.nanolett.5b00133.
- [162] N. M. Andoy, M. S. Filipiak, D. Vetter, Ó. Gutiérrez-Sanz, and A. Tarasov, 'Graphene-Based Electronic Immunosensor with Femtomolar Detection Limit in Whole Serum', *Adv. Mater. Technol.*, vol. 3, no. 12, p. 1800186, Dec. 2018, doi: 10.1002/admt.201800186.
- [163] N. Haustein, Ó. Gutiérrez-Sanz, and A. Tarasov, 'Analytical Model To Describe the Effect of Polyethylene Glycol on Ionic Screening of Analyte Charges in Transistor-Based Immunosensing', *ACS Sens.*, vol. 4, no. 4, pp. 874–882, Apr. 2019, doi: 10.1021/acssensors.8b01515.
- [164] R. B. M. Schasfoort, P. Bergveld, R. P. H. Kooyman, and J. Greve, 'Possibilities and limitations of direct detection of protein charges by means of an immunological field-effect transistor', *Anal. Chim. Acta*, vol. 238, pp. 323–329, 1990, doi: 10.1016/S0003-2670(00)80554-1.
- [165] S.-K. Hu, F.-Y. Lo, Chia Chien Hsieh, and L. Chao, 'Sensing Ability and Formation Criterion of Fluid Supported Lipid Bilayer Coated Graphene Field-Effect Transistors', *ACS Sens.*, vol. 4, no. 4, pp. 892–899, 2019, doi: 10.1021/acssensors.8b01623.
- [166] A. Romagnoli *et al.*, 'SARS-CoV-2 multi-variant rapid detector based on graphene transistor functionalized with an engineered dimeric ACE2 receptor', *Nano Today*, vol. 48, p. 101729, 2023, doi: 10.1016/j.nantod.2022.101729.
- [167] M. S. Filipiak *et al.*, 'Highly sensitive, selective and label-free protein detection in physiological solutions using carbon nanotube transistors with nanobody receptors', *Sens. Actuators B Chem.*, vol. 255, pp. 1507–1516, 2018, doi: 10.1016/j.snb.2017.08.164.
- [168] M. Hosseindokht, H. Bakherad, and H. Zare, 'Nanobodies: a tool to open new horizons in diagnosis and treatment of prostate cancer', *Cancer Cell Int.*, vol. 21, no. 1, 2021, doi: 10.1186/s12935-021-02285-0.
- [169] J. Zhang, H. Sun, W. Pei, H. Jiang, and J. Chen, 'Nanobody-based immunosensing methods for safeguarding public health', *J. Biomed. Res.*, vol. 35, no. 4, pp. 318–326, 2021, doi: 10.7555/JBR.35.20210108.

-
- [170] D. Wu, Y. Yu, D. Jin, M. Xiao, Z. Zhang, and G.-J. Zhang, 'Dual-Aptamer Modified Graphene Field-Effect Transistor Nanosensor for Label-Free and Specific Detection of Hepatocellular Carcinoma-Derived Microvesicles', vol. 92, no. 5, pp. 4006–4015, 2020, doi: 10.1021/acs.analchem.9b05531.
- [171] S. Cai, J. Yan, H. Xiong, Y. Liu, D. Peng, and Z. Liu, 'Investigations on the interface of nucleic acid aptamers and binding targets', *The Analyst*, vol. 143, no. 22, pp. 5317–5338, 2018, doi: 10.1039/c8an01467a.
- [172] C. Tuerk and L. Gold, 'Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase', *Science*, vol. 249, no. 4968, pp. 505–510, 1990, doi: 10.1126/science.2200121.
- [173] A. V. Lakhin, V. Z. Tarantul, and L. V. Gening, 'Aptamers: Problems, Solutions and Prospects', *Acta Naturae*, vol. 5, no. 4, pp. 34–43, 2013.
- [174] M. Tian *et al.*, 'Highly-sensitive graphene field effect transistor biosensor using PNA and DNA probes for RNA detection', *Appl. Surf. Sci.*, vol. 527, p. 146839, 2020, doi: 10.1016/j.apsusc.2020.146839.
- [175] J. Li *et al.*, 'Rapid and unamplified identification of COVID-19 with morpholino-modified graphene field-effect transistor nanosensor', *Biosens. Bioelectron.*, vol. 183, p. 113206, 2021, doi: 10.1016/j.bios.2021.113206.
- [176] B. Cai, S. Wang, L. Huang, Y. Ning, Z. Zhang, and G.-J. Zhang, 'Ultrasensitive Label-Free Detection of PNA–DNA Hybridization by Reduced Graphene Oxide Field-Effect Transistor Biosensor', *ACS Nano*, vol. 8, no. 3, pp. 2632–2638, 2014, doi: 10.1021/nn4063424.
- [177] X. Wang, C. Dai, Y. Wu, Y. Liu, and D. Wei, 'Molecular-electromechanical system for unamplified detection of trace analytes in biofluids', *Nat. Protoc.*, pp. 1–41, 2023, doi: 10.1038/s41596-023-00830-x.
- [178] L. Wang *et al.*, 'Rapid and ultrasensitive electromechanical detection of ions, biomolecules and SARS-CoV-2 RNA in unamplified samples', *Nat. Biomed. Eng.*, vol. 6, no. 3, pp. 276–285, 2022, doi: 10.1038/s41551-021-00833-7.
- [179] J. Rexha *et al.*, 'Unlocking the Potential of Field Effect Transistor (FET) Biosensors: A Perspective on Methodological Advances in Computational and Molecular Biology', *Adv. Sens. Res.*, vol. 2, no. 11, 2023, doi: 10.1002/adsr.202300053.
- [180] Y. Wu *et al.*, 'Triple-Probe DNA Framework-Based Transistor for SARS-CoV-2 10-in-1 Pooled Testing', *Nano Lett.*, vol. 22, no. 8, pp. 3307–3316, Apr. 2022, doi: 10.1021/acs.nanolett.2c00415.
- [181] S. Balderston *et al.*, 'Discrimination of single-point mutations in unamplified genomic DNA via Cas9 immobilized on a graphene field-effect transistor', *Nat. Biomed. Eng.*, vol. 5, no. 7, pp. 713–725, Apr. 2021, doi: 10.1038/s41551-021-00706-z.
- [182] J. Li *et al.*, 'Tandem Cas13a/crRNA-Mediated CRISPR-FET Biosensor: A One-for-All Check Station for Virus without Amplification', *ACS Sens.*, vol. 7, no. 9, pp. 2680–2690, Sep. 2022, doi: 10.1021/acssensors.2c01200.

-
- [183] R. Hajian *et al.*, 'Detection of unamplified target genes via CRISPR–Cas9 immobilized on a graphene field-effect transistor', *Nat. Biomed. Eng.*, vol. 3, no. 6, pp. 427–437, Jun. 2019, doi: 10.1038/s41551-019-0371-x.
- [184] G. S. Kulkarni and Z. Zhong, 'Detection beyond the Debye Screening Length in a High-Frequency Nanoelectronic Biosensor', *Nano Lett.*, vol. 12, no. 2, pp. 719–723, Feb. 2012, doi: 10.1021/nl203666a.
- [185] W. Fu *et al.*, 'Electrolyte gate dependent high-frequency measurement of graphene field-effect transistor for sensing applications', *Appl. Phys. Lett.*, vol. 104, no. 1, p. 013102, Jan. 2014, doi: 10.1063/1.4857616.
- [186] J. O. Bockris, E. Gileadi, and K. Müller, 'Dielectric Relaxation in the Electric Double Layer', *J. Chem. Phys.*, vol. 44, no. 4, pp. 1445–1456, Feb. 1966, doi: 10.1063/1.1726876.
- [187] X. Zhang *et al.*, 'Dielectric-Modulated Biosensing with Ultrahigh-Frequency-Operated Graphene Field-Effect Transistors', *Adv. Mater.*, vol. 34, no. 7, p. 2106666, Feb. 2022, doi: 10.1002/adma.202106666.
- [188] G. S. Kulkarni, K. Reddy, Z. Zhong, and X. Fan, 'Graphene nanoelectronic heterodyne sensor for rapid and sensitive vapour detection', *Nat. Commun.*, vol. 5, no. 1, pp. 1–7, 2014, doi: 10.1038/ncomms5376.
- [189] P. R. Nair and M. A. Alam, 'Theory of "Selectivity" of label-free nanobiosensors: A geometro-physical perspective', *J. Appl. Phys.*, vol. 107, no. 6, p. 064701, Mar. 2010, doi: 10.1063/1.3310531.
- [190] A. Frutiger, A. Tanno, S. Hwu, R. F. Tiefenauer, J. Vörös, and N. Nakatsuka, 'Nonspecific Binding—Fundamental Concepts and Consequences for Biosensing Applications', *Chem. Rev.*, vol. 121, no. 13, pp. 8095–8160, Jul. 2021, doi: 10.1021/acs.chemrev.1c00044.
- [191] R. Campos *et al.*, 'Attomolar Label-Free Detection of DNA Hybridization with Electrolyte-Gated Graphene Field-Effect Transistors', *ACS Sens.*, vol. 4, no. 2, pp. 286–293, Feb. 2019, doi: 10.1021/acssensors.8b00344.
- [192] E. Fernandes *et al.*, 'Functionalization of single-layer graphene for immunoassays', *Appl. Surf. Sci.*, vol. 480, pp. 709–716, Jun. 2019, doi: 10.1016/j.apsusc.2019.03.004.
- [193] L. Xu *et al.*, 'Detection of Glial Fibrillary Acidic Protein in Patient Plasma Using On-Chip Graphene Field-Effect Biosensors, in Comparison with ELISA and Single-Molecule Array', *ACS Sens.*, vol. 7, no. 1, pp. 253–262, 2021, doi: 10.1021/acssensors.1c02232.
- [194] L. Zhou *et al.*, 'Novel Graphene Biosensor Based on the Functionalization of Multifunctional Nanobovine Serum Albumin for the Highly Sensitive Detection of Cancer Biomarkers', *Nano-Micro Lett.*, vol. 11, no. 1, p. 20, Dec. 2019, doi: 10.1007/s40820-019-0250-8.
- [195] N. Talib, F. Salam, and Y. Sulaiman, 'Development of Highly Sensitive Immunosensor for Clenbuterol Detection by Using Poly(3,4-ethylenedioxythiophene)/Graphene Oxide Modified Screen-Printed Carbon Electrode', *Sensors*, vol. 18, no. 12, p. 4324, Dec. 2018, doi: 10.3390/s18124324.

-
- [196] X. Wang, Z. Hao, T. R. Olsen, W. Zhang, and Q. Lin, 'Measurements of aptamer–protein binding kinetics using graphene field-effect transistors', p. 9, 2019.
- [197] O. Oshin, D. Kireev, H. Hlukhova, F. Idachaba, D. Akinwande, and A. Atayero, 'Graphene-Based Biosensor for Early Detection of Iron Deficiency', p. 13, 2020.
- [198] M. Abrantes *et al.*, 'Ultrasensitive dopamine detection with graphene aptasensor multitransistor arrays', *J. Nanobiotechnology*, vol. 20, no. 1, p. 495, Nov. 2022, doi: 10.1186/s12951-022-01695-0.
- [199] 'Novel graphene-based biosensor for early detection of Zika virus infection', *Biosens. Bioelectron.*, vol. 100, pp. 85–88, 2018, doi: 10.1016/j.bios.2017.08.051.
- [200] Z. Wang *et al.*, 'An Ultraflexible and Stretchable Aptameric Graphene Nanosensor for Biomarker Detection and Monitoring', *Adv. Funct. Mater.*, vol. 29, no. 44, p. 1905202, 2019, doi: 10.1002/adfm.201905202.
- [201] S. Ghosh, N. I. Khan, J. G. Tsavalas, and E. Song, 'Selective Detection of Lysozyme Biomarker Utilizing Large Area Chemical Vapor Deposition-Grown Graphene-Based Field-Effect Transistor', *Front. Bioeng. Biotechnol.*, vol. 6, 2018, doi: 10.3389/fbioe.2018.00029.
- [202] Y. Kumada, 'Site-specific immobilization of recombinant antibody fragments through material-binding peptides for the sensitive detection of antigens in enzyme immunoassays', *Biochim. Biophys. Acta*, p. 10, 1844.
- [203] H.-W. Lu *et al.*, 'The promise of graphene-based transistors for democratizing multiomics studies', *Biosens. Bioelectron.*, vol. 195, p. 113605, Jan. 2022, doi: 10.1016/j.bios.2021.113605.
- [204] B. Thakur, 'Rapid detection of single E. coli bacteria using a graphene-based field-effect transistor device', *Biosens. Bioelectron.*, p. 7, 2018.
- [205] J. Chang *et al.*, 'Real-time detection of mercury ions in water using a reduced graphene oxide/DNA field-effect transistor with assistance of a passivation layer', *Sens. Bio-Sens. Res.*, vol. 5, pp. 97–104, Sep. 2015, doi: 10.1016/j.sbsr.2015.07.009.
- [206] I. Fakih, F. Mahvash, M. Siaj, and T. Szkopek, 'Sensitive Precise p H Measurement with Large-Area Graphene Field-Effect Transistors at the Quantum-Capacitance Limit', *Phys. Rev. Appl.*, vol. 8, no. 4, p. 044022, Oct. 2017, doi: 10.1103/PhysRevApplied.8.044022.
- [207] L. Yang *et al.*, 'Antifouling Field-Effect Transistor Sensing Interface Based on Covalent Organic Frameworks', *Adv. Electron. Mater.*, vol. 6, no. 5, p. 1901169, May 2020, doi: 10.1002/aelm.201901169.
- [208] P. Karnatak, T. P. Sai, S. Goswami, S. Ghatak, S. Kaushal, and A. Ghosh, 'Current crowding mediated large contact noise in graphene field-effect transistors', *Nat. Commun.*, vol. 7, no. 1, p. 13703, Dec. 2016, doi: 10.1038/ncomms13703.
- [209] H. N. Arnold *et al.*, 'Reducing flicker noise in chemical vapor deposition graphene field-effect transistors', *Appl. Phys. Lett.*, vol. 108, no. 7, p. 073108, Feb. 2016, doi: 10.1063/1.4942468.

-
- [210] T. Manimekala, R. Sivasubramanian, and G. Dharmalingam, 'Nanomaterial-Based Biosensors using Field-Effect Transistors: A Review', *J. Electron. Mater.*, vol. 51, no. 5, pp. 1950–1973, May 2022, doi: 10.1007/s11664-022-09492-z.
- [211] P. Karnatak, T. Paul, S. Islam, and A. Ghosh, '1 / f noise in van der Waals materials and hybrids', *Adv. Phys. X*, vol. 2, no. 2, pp. 428–449, Mar. 2017, doi: 10.1080/23746149.2017.1314192.
- [212] D. Kireev *et al.*, 'Graphene transistors for interfacing with cells: towards a deeper understanding of liquid gating and sensitivity', *Sci. Rep.*, vol. 7, no. 1, p. 6658, Dec. 2017, doi: 10.1038/s41598-017-06906-5.
- [213] A. Rehman *et al.*, 'Nature of the 1/ f noise in graphene—direct evidence for the mobility fluctuation mechanism', *Nanoscale*, vol. 14, no. 19, pp. 7242–7249, 2022, doi: 10.1039/D2NR00207H.
- [214] W. Fu *et al.*, 'Biosensing near the neutrality point of graphene', *Sci. Adv.*, vol. 3, no. 10, p. e1701247, Oct. 2017, doi: 10.1126/sciadv.1701247.
- [215] S. Peng *et al.*, 'Carrier-Number-Fluctuation Induced Ultralow 1/ f Noise Level in Top-Gated Graphene Field Effect Transistor', *ACS Appl. Mater. Interfaces*, vol. 9, no. 8, pp. 6661–6665, Mar. 2017, doi: 10.1021/acsami.6b15862.
- [216] S. Jiang *et al.*, 'Real-time electrical detection of nitric oxide in biological systems with sub-nanomolar sensitivity', *Nat. Commun.*, vol. 4, no. 1, p. 2225, 2013, doi: 10.1038/ncomms3225.
- [217] H. Xie *et al.*, 'Real-Time Monitoring of Nitric Oxide at Single-Cell Level with Porphyrin-Functionalized Graphene Field-Effect Transistor Biosensor', *Anal. Chem.*, vol. 88, no. 22, pp. 11115–11122, 2016, doi: 10.1021/acs.analchem.6b03208.
- [218] Y.-M. Lei *et al.*, 'Detection of heart failure-related biomarker in whole blood with graphene field effect transistor biosensor', *Biosens. Bioelectron.*, vol. 91, pp. 1–7, 2017, doi: 10.1016/j.bios.2016.12.018.
- [219] Kyung Hwa Cho, D. M. Shin, J. Oh, Ji Yeong An, Jun Yeob Lee, and J. Jang, 'Multidimensional Conductive Nanofilm-Based Flexible Aptasensor for Ultrasensitive and Selective HBsAg Detection', vol. 10, no. 34, pp. 28412–28419, 2018, doi: 10.1021/acsami.8b09918.
- [220] W.-M. Munief *et al.*, 'Reduced graphene oxide biosensor platform for the detection of NT-proBNP biomarker in its clinical range', *Biosens. Bioelectron.*, vol. 126, pp. 136–142, 2019, doi: 10.1016/j.bios.2018.09.102.
- [221] J. Gao *et al.*, 'Poly-L-Lysine-Modified Graphene Field-Effect Transistor Biosensors for Ultrasensitive Breast Cancer miRNAs and SARS-CoV-2 RNA Detection', vol. 94, no. 3, pp. 1626–1636, 2022, doi: 10.1021/acs.analchem.1c03786.
- [222] H.-J. Jang *et al.*, 'Remote Floating-Gate Field-Effect Transistor with 2-Dimensional Reduced Graphene Oxide Sensing Layer for Reliable Detection of SARS-CoV-2 Spike Proteins', vol. 14, no. 21, pp. 24187–24196, 2022, doi: 10.1021/acsami.2c04969.

-
- [223] L. Xu *et al.*, 'On-chip integrated graphene aptasensor with portable readout for fast and label-free COVID-19 detection in virus transport medium', *Sens. Diagn.*, 2022, doi: 10.1039/d2sd00076h.
- [224] 'The Olympus BX51 WI', The Olympus BX51 WI. Accessed: Jan. 01, 2023. [Online]. Available: <https://www.scientifica.uk.com/products/olympus-bx51-wi#>
- [225] D. Nečas and P. Klapetek, 'Gwyddion: an open-source software for SPM data analysis', 2011. Accessed: Jan. 01, 2022. [Online]. Available: <https://www.degruyter.com/document/doi/10.2478/s11534-011-0096-2/html>
- [226] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, 'NIH Image to ImageJ: 25 years of image analysis', *Nat. Methods*, vol. 9, no. 7, pp. 671–675, 2012, doi: 10.1038/nmeth.2089.
- [227] F. de Chaumont *et al.*, 'Computerized video analysis of social interactions in mice', *Nat. Methods*, vol. 9, no. 4, pp. 410–417, 2012, doi: 10.1038/nmeth.1924.
- [228] J.-C. Olivo-Marin, 'Extraction of spots in biological images using multiscale products', *Pattern Recognit.*, vol. 35, no. 9, pp. 1989–1996, 2002, doi: 10.1016/s0031-3203(01)00127-3.
- [229] A. C. Ferrari and D. M. Basko, 'Raman spectroscopy as a versatile tool for studying the properties of graphene', *Nat. Nanotechnol.*, vol. 8, no. 4, pp. 235–246, 2013, doi: 10.1038/nnano.2013.46.
- [230] L. Xu *et al.*, 'On-chip integrated graphene aptasensor with portable readout for fast and label-free COVID-19 detection in virus transport medium', *Sens. Diagn.*, p. 10.1039.D2SD00076H, 2022, doi: 10.1039/D2SD00076H.
- [231] P. Blaha *et al.*, *WIEN2k: An Augmented Plane Wave Plus Local Orbitals Program for Calculating Crystal Properties*. in Northwestern Scholars. Techn. Universitat, 2019. Accessed: Jan. 01, 2023. [Online]. Available: <https://www.scholars.northwestern.edu/en/publications/wien2k-an-augmented-plane-wave-plus-local-orbitals-program-for-ca>
- [232] J. P. Perdew, K. Burke, and M. Ernzerhof, 'Generalized Gradient Approximation Made Simple', *Phys. Rev. Lett.*, vol. 77, no. 18, pp. 3865–3868, 1996, doi: 10.1103/physrevlett.77.3865.
- [233] G. Kresse and D. Joubert, 'From ultrasoft pseudopotentials to the projector augmented-wave method', *Phys. Rev. B*, vol. 59, no. 3, pp. 1758–1775, 1999, doi: 10.1103/physrevb.59.1758.
- [234] V. I. Anisimov, J. Zaanen, and Ole K  seler Andersen, 'Band theory and Mott insulators: HubbardUinstead of StonerI', *Phys. Rev.*, vol. 44, no. 3, pp. 943–954, 1991, doi: 10.1103/physrevb.44.943.
- [235] S. Grimme, J. Antony, S. Ehrlich, and H. Krieg, 'A consistent and accurate ab initio parametrization of density functional dispersion correction (DFT-D) for the 94 elements H-Pu', *J. Chem. Phys.*, vol. 132, no. 15, p. 154104, 2010, doi: 10.1063/1.3382344.
- [236] X. Luo and J. J. Davis, 'Electrical biosensors and the label free detection of protein disease biomarkers', *Chem. Soc. Rev.*, vol. 42, no. 13, p. 5944, 2013, doi: 10.1039/c3cs60077g.

-
- [237] G. Seo *et al.*, 'Rapid Detection of COVID-19 Causative Virus (SARS-CoV-2) in Human Nasopharyngeal Swab Specimens Using Field-Effect Transistor-Based Biosensor', *ACS Nano*, vol. 14, no. 4, pp. 5135–5142, 2020, doi: 10.1021/acsnano.0c02823.
- [238] Sung Yun Woo *et al.*, 'Protein Detection Using Quadratic Fit Analysis near the Dirac Point of Graphene Field-Effect Biosensors', vol. 2, no. 4, pp. 913–919, 2020, doi: 10.1021/acsaelm.9b00840.
- [239] R. Ge, X. Li, S.-Z. Kang, L. Qin, and G. Li, 'Highly efficient graphene oxide/porphyrin photocatalysts for hydrogen evolution and the interfacial electron transfer', *Appl. Catal. B Environ.*, vol. 187, pp. 67–74, 2016, doi: 10.1016/j.apcatb.2016.01.024.
- [240] M. Zhu *et al.*, 'Surfactant Assistance in Improvement of Photocatalytic Hydrogen Production with the Porphyrin Noncovalently Functionalized Graphene Nanocomposite', *ACS Appl. Mater. Interfaces*, vol. 5, no. 5, pp. 1732–1740, 2013, doi: 10.1021/am302912v.
- [241] B. Mao, B. J. Hodges, C. Franklin, D. G. Calatayud, and S. I. Pascu, 'Self-Assembled Materials Incorporating Functional Porphyrins and Carbon Nanoplateforms as Building Blocks for Photovoltaic Energy Applications', vol. 9, 2021, doi: 10.3389/fchem.2021.727574.
- [242] S.-J. Kim *et al.*, 'Tunable functionalization of graphene nanosheets for graphene-organic hybrid photodetectors', vol. 27, no. 7, pp. 075709–075709, 2016, doi: 10.1088/0957-4484/27/7/075709.
- [243] Y. Yang, 'Porphyrin Functionalized Graphene for Sensitive Electrochemical Detection of Uric Acid', *Int. J. Electrochem. Sci.*, pp. 7370–7379, 2016, doi: 10.20964/2016.09.41.
- [244] A. Lewandowska-Andralojc *et al.*, 'Understanding structure–properties relationships of porphyrin linked to graphene oxide through π – π -stacking or covalent amide bonds', *Sci. Rep.*, vol. 12, no. 1, p. 13420, 2022, doi: 10.1038/s41598-022-16931-8.
- [245] H. Zhang, Y. Han, Y. Guo, and C. Dong, 'Porphyrin functionalized graphene nanosheets-based electrochemical aptasensor for label-free ATP detection', *J. Mater. Chem.*, vol. 22, no. 45, p. 23900, 2012, doi: 10.1039/c2jm35379b.
- [246] J. Liu *et al.*, 'The Dependence of Graphene Raman D-band on Carrier Density', *Nano Lett.*, vol. 13, no. 12, pp. 6170–6175, 2013, doi: 10.1021/nl4035048.
- [247] C.-F. Chen *et al.*, 'Controlling inelastic light scattering quantum pathways in graphene', *Nature*, vol. 471, no. 7340, pp. 617–620, 2011, doi: 10.1038/nature09866.
- [248] T. M. Cotton, S. G. Schultz, and R. P. Van Duyne, 'Surface-enhanced resonance Raman scattering from water-soluble porphyrins adsorbed on a silver electrode', *J. Am. Chem. Soc.*, vol. 104, no. 24, pp. 6528–6532, 1982, doi: 10.1021/ja00388a008.

-
- [249] B. S. McAvan *et al.*, 'Raman Spectroscopy to Monitor Post-Translational Modifications and Degradation in Monoclonal Antibody Therapeutics', *Anal. Chem.*, vol. 92, no. 15, pp. 10381–10389, 2020, doi: 10.1021/acs.analchem.0c00627.
- [250] G. V. Barnett *et al.*, 'Structural Changes and Aggregation Mechanisms for Anti-Streptavidin IgG1 at Elevated Concentration', *J. Phys. Chem. B*, vol. 119, no. 49, pp. 15150–15163, 2015, doi: 10.1021/acs.jpcc.5b08748.
- [251] G. de, R. Goodacre, and L. Ashton, 'Monitoring Antibody Aggregation in Early Drug Development Using Raman Spectroscopy and Perturbation-Correlation Moving Windows', *Anal. Chem.*, vol. 86, no. 22, pp. 11133–11140, 2014, doi: 10.1021/ac5038329.
- [252] I. Ettah and L. Ashton, 'Engaging with Raman Spectroscopy to Investigate Antibody Aggregation', *Antibodies*, vol. 7, no. 3, p. 24, 2018, doi: 10.3390/antib7030024.
- [253] Malkolm Hinnemo *et al.*, 'On Monolayer Formation of Pyrenebutyric Acid on Graphene', *Langmuir*, vol. 33, no. 15, pp. 3588–3593, 2017, doi: 10.1021/acs.langmuir.6b04237.
- [254] H. Wang, D. G. Castner, B. D. Ratner, and S. Jiang, 'Probing the Orientation of Surface-Immobilized Immunoglobulin G by Time-of-Flight Secondary Ion Mass Spectrometry', *Langmuir*, vol. 20, no. 5, pp. 1877–1887, 2004, doi: 10.1021/la035376f.
- [255] F. Liu, Manish Kumar Dubey, H. Takahashi, D. G. Castner, and D. W. Grainger, 'Immobilized Antibody Orientation Analysis Using Secondary Ion Mass Spectrometry and Fluorescence Imaging of Affinity-Generated Patterns', vol. 82, no. 7, pp. 2947–2958, 2010, doi: 10.1021/ac902964q.
- [256] V. Lebec, Souhir Boujday, C. Poleunis, C. Pradier, and Arnaud Delcorte, 'Time-of-Flight Secondary Ion Mass Spectrometry Investigation of the Orientation of Adsorbed Antibodies on SAMs Correlated to Biorecognition Tests', vol. 118, no. 4, pp. 2085–2092, 2014, doi: 10.1021/jp410845g.
- [257] N. G. Welch *et al.*, 'Surface immobilized antibody orientation determined using ToF-SIMS and multivariate analysis', *Acta Biomater.*, vol. 55, pp. 172–182, 2017, doi: 10.1016/j.actbio.2017.03.038.
- [258] K. Gajos *et al.*, 'Indirect immunoassay on functionalized silicon surface: Molecular arrangement, composition and orientation examined step-by-step with multi-technique and multivariate analysis', *Colloids Surf. B Biointerfaces*, vol. 150, pp. 437–444, 2017, doi: 10.1016/j.colsurfb.2016.11.009.
- [259] K. Awsiuk *et al.*, 'Orientation and biorecognition of immunoglobulin adsorbed on spin-cast poly(3-alkylthiophenes): Impact of polymer film crystallinity', *Colloids Surf. B Biointerfaces*, vol. 148, pp. 278–286, 2016, doi: 10.1016/j.colsurfb.2016.08.028.
- [260] Y.-P. Kim *et al.*, 'Quantitative Analysis of Surface-Immobilized Protein by TOF-SIMS: Effects of Protein Orientation and Trehalose Additive', *Anal. Chem.*, vol. 79, no. 4, pp. 1377–1385, 2007, doi: 10.1021/ac0616005.

-
- [261] N. G. Welch, R. M. T. Madiona, J. A. Scoble, B. W. Muir, and P. J. Pigram, 'ToF-SIMS and Principal Component Analysis Investigation of Denatured, Surface-Adsorbed Antibodies', *Langmuir*, vol. 32, no. 42, pp. 10824–10834, 2016, doi: 10.1021/acs.langmuir.6b02754.
- [262] S. Wold, K. Esbensen, and P. Geladi, 'Principal component analysis', *Chemom. Intell. Lab. Syst.*, vol. 2, no. 1–3, pp. 37–52, 1987, doi: 10.1016/0169-7439(87)80084-9.
- [263] R. P. D. Bank, 'RCSB PDB - 4HBC: Crystal structure of a conformation-dependent rabbit IgG Fab specific for amyloid prefibrillar oligomers', www.rcsb.org. Accessed: Jan. 01, 2023. [Online]. Available: <https://www.rcsb.org/structure/4HBC>
- [264] R. P. D. Bank, 'RCSB PDB - 2VUO: Crystal structure of the rabbit IgG Fc fragment', www.rcsb.org. Accessed: Jan. 01, 2023. [Online]. Available: <https://www.rcsb.org/structure/2VUO>
- [265] N. T. Samuel, M. Wagner, K. D. Dornfeld, and D. G. Castner, 'Analysis of Poly(amino acids) by Static Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS)', vol. 8, no. 3, pp. 163–184, 2001, doi: 10.1116/11.20020301.
- [266] F. Pedregosa *et al.*, 'Scikit-learn: Machine Learning in Python', *J Mach Learn Res*, 2011, doi: 10.5555/1953048.2078195.
- [267] V. A. Karachevtsev *et al.*, 'Noncovalent Interaction of Single-Walled Carbon Nanotubes with 1-Pyrenebutanoic Acid Succinimide Ester and Glucoseoxidase', vol. 115, no. 43, pp. 21072–21082, 2011, doi: 10.1021/jp207916d.
- [268] V. A. Karachevtsev, S. G. Stepanian, M. V. Karachevtsev, and L. Adamowicz, 'Graphene induced molecular flattening of meso-5,10,15,20-tetraphenyl porphyrin: DFT calculations and molecular dynamics simulations', *Comput. Theor. Chem.*, vol. 1133, pp. 1–6, 2018, doi: 10.1016/j.comptc.2018.04.009.
- [269] J. Touzeau, F. Barbault, F. Maurel, and M. Seydou, 'Insights on porphyrin-functionalized graphene: Theoretical study of substituent and metal-center effects on adsorption', *Chem. Phys. Lett.*, vol. 713, pp. 172–179, 2018, doi: 10.1016/j.cplett.2018.10.046.
- [270] Y. Xu, L. Zhao, H. Bai, W. Hong, C. Li, and G. Shi, 'Chemically Converted Graphene Induced Molecular Flattening of 5,10,15,20-Tetrakis(1-methyl-4-pyridinio)porphyrin and Its Application for Optical Detection of Cadmium(II) Ions', *J. Am. Chem. Soc.*, vol. 131, no. 37, pp. 13490–13497, 2009, doi: 10.1021/ja905032g.
- [271] Y. Ishida, D. Masui, T. Shimada, H. Tachibana, H. Inoue, and S. Takagi, 'The Mechanism of the Porphyrin Spectral Shift on Inorganic Nanosheets: The Molecular Flattening Induced by the Strong Host–Guest Interaction due to the "Size-Matching Rule"', vol. 116, no. 14, pp. 7879–7885, 2012, doi: 10.1021/jp300842f.
- [272] S. M. Aly, M. R. Parida, E. Alarousu, and O. F. Mohammed, 'Ultrafast electron injection at the cationic porphyrin–graphene interface assisted by molecular flattening', *Chem. Commun.*, vol. 50, no. 72, p. 10452, 2014, doi: 10.1039/c4cc04985c.

-
- [273] V. A. Karachevtsev, S. G. Stepanian, M. V. Karachevtsev, V. A. Valeev, and L. Adamowicz, 'Structural and spectral transformation of cationic porphyrin TMPyP4 at adsorption on graphene', *J. Mol. Struct.*, vol. 1245, p. 131056, 2021, doi: 10.1016/j.molstruc.2021.131056.
- [274] R. Kalluri and V. S. LeBleu, 'The biology, function, and Biomedical Applications of Exosomes', *Science*, vol. 367, no. 6478, 2020, doi: 10.1126/science.aau6977.
- [275] L. Doyle and M. Wang, 'Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis', *Cells*, vol. 8, no. 7, p. 727, 2019, doi: 10.3390/cells8070727.
- [276] D. Burtenshaw *et al.*, 'Exosomal Composition, Biogenesis and Profiling Using Point-of-Care Diagnostics—Implications for Cardiovascular Disease', *Front. Cell Dev. Biol.*, vol. 10, 2022, doi: 10.3389/fcell.2022.853451.
- [277] W. Wang *et al.*, 'The crosstalk: exosomes and lipid metabolism', vol. 18, no. 1, 2020, doi: 10.1186/s12964-020-00581-2.
- [278] Y. Zhang, Y. Liu, H. Liu, and W. H. Tang, 'Exosomes: biogenesis, biologic function and clinical potential', *Cell Biosci.*, vol. 9, no. 1, 2019, doi: 10.1186/s13578-019-0282-2.
- [279] M. Groot and H. Lee, 'Sorting Mechanisms for MicroRNAs into Extracellular Vesicles and Their Associated Diseases', *Cells*, vol. 9, no. 4, p. 1044, 2020, doi: 10.3390/cells9041044.
- [280] S. Tan *et al.*, 'Exosomal miRNAs in tumor microenvironment', *J. Exp. Clin. Cancer Res.*, vol. 39, no. 1, 2020, doi: 10.1186/s13046-020-01570-6.
- [281] L. Pascucci *et al.*, 'Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit in vitro tumor growth: A new approach for drug delivery', *J. Controlled Release*, vol. 192, pp. 262–270, 2014, doi: 10.1016/j.jconrel.2014.07.042.
- [282] S. S. Kanwar, C. J. Dunlay, D. M. Simeone, and S. Negrath, 'Microfluidic device (ExoChip) for on-chip isolation, quantification and characterization of circulating exosomes', *Lab Chip*, vol. 14, no. 11, pp. 1891–1900, 2014, doi: 10.1039/c4lc00136b.
- [283] S. A. Melo *et al.*, 'Glypican-1 identifies cancer exosomes and detects early pancreatic cancer', *Nature*, vol. 523, no. 7559, pp. 177–182, 2015, doi: 10.1038/nature14581.
- [284] C. Hu *et al.*, 'Potentiality of Exosomal Proteins as Novel Cancer Biomarkers for Liquid Biopsy', *Front. Immunol.*, vol. 13, 2022, doi: 10.3389/fimmu.2022.792046.
- [285] S. Cherevko, A. A. Topalov, A. R. Zeradjanin, I. Katsounaros, and K. J. J. Mayrhofer, 'Gold dissolution: towards understanding of noble metal corrosion', *RSC Adv.*, vol. 3, no. 37, p. 16516, 2013, doi: 10.1039/c3ra42684j.
- [286] C. Liu *et al.*, 'Single-Exosome-Counting Immunoassays for Cancer Diagnostics', *Nano Lett.*, vol. 18, no. 7, pp. 4226–4232, Jul. 2018, doi: 10.1021/acs.nanolett.8b01184.

-
- [287] S. A. Melo *et al.*, 'Glypican-1 identifies cancer exosomes and detects early pancreatic cancer', *Nature*, vol. 523, no. 7559, pp. 177–182, 2015, doi: 10.1038/nature14581.
- [288] K. Matsuda *et al.*, 'Glypican-1 is overexpressed in human breast cancer and modulates the mitogenic effects of multiple heparin-binding growth factors in breast cancer cells.', *Cancer Res.*, vol. 61, no. 14, pp. 5562–5569, 2001.
- [289] J. Kleeff *et al.*, 'The cell-surface heparan sulfate proteoglycan glypican-1 regulates growth factor action in pancreatic carcinoma cells and is overexpressed in human pancreatic cancer.', www.jci.org. Accessed: Jan. 01, 2023. [Online]. Available: <https://www.jci.org/articles/view/4105/pdf>
- [290] H. Etayash, A. R. McGee, K. Kaur, and T. Thundat, 'Nanomechanical sandwich assay for multiple cancer biomarkers in breast cancer cell-derived exosomes', *Nanoscale*, vol. 8, no. 33, pp. 15137–15141, 2016, doi: 10.1039/C6NR03478K.
- [291] X. Wang, J. Huang, W. Chen, G. Li, Z. Li, and J. Lei, 'The updated role of exosomal proteins in the diagnosis, prognosis, and treatment of cancer', *Exp. Mol. Med.*, vol. 54, no. 9, pp. 1390–1400, 2022, doi: 10.1038/s12276-022-00855-4.
- [292] Z. Yu, Y. Yang, W. Fang, P. Hu, Y. Liu, and J. Shi, 'Dual Tumor Exosome Biomarker Co-recognitions Based Nanoliquid Biopsy for the Accurate Early Diagnosis of Pancreatic Cancer', 2023, doi: 10.1021/acsnano.3c00674.
- [293] H. Sung *et al.*, 'Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries', *CA. Cancer J. Clin.*, vol. 71, no. 3, pp. 209–249, 2021, doi: 10.3322/caac.21660.
- [294] J. Yang, R. Xu, C. Wang, J. Qiu, B. Ren, and L. You, 'Early screening and diagnosis strategies of pancreatic cancer: a comprehensive review', *Cancer Commun.*, 2021, doi: 10.1002/cac2.12204.
- [295] G. Gheorghe *et al.*, 'Early Diagnosis of Pancreatic Cancer: The Key for Survival', *Diagnostics*, vol. 10, no. 11, p. 869, 2020, doi: 10.3390/diagnostics10110869.
- [296] E. Heitzer, I. S. Haque, C. E. S. Roberts, and M. R. Speicher, 'Current and future perspectives of liquid biopsies in genomics-driven oncology', *Nat. Rev. Genet.*, vol. 20, no. 2, pp. 71–88, 2018, doi: 10.1038/s41576-018-0071-5.
- [297] J. D. Cohen *et al.*, 'Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers', *Proc. Natl. Acad. Sci. U. S. A.*, vol. 114, no. 38, pp. 10202–10207, 2017, doi: 10.1073/pnas.1704961114.
- [298] L. Sivapalan, H. M. Kocher, H. Ross-Adams, and C. Chelala, 'Molecular profiling of ctDNA in pancreatic cancer: Opportunities and challenges for clinical application', *Pancreatology*, 2020, doi: 10.1016/j.pan.2020.12.017.

-
- [299] B. G. Song *et al.*, 'Detection of Circulating Tumor Cells in Resectable Pancreatic Ductal Adenocarcinoma: A Prospective Evaluation as a Prognostic Marker', *Front. Oncol.*, vol. 10, p. 616440, 2020, doi: 10.3389/fonc.2020.616440.
- [300] J. P. Hinestrosa *et al.*, 'Early-stage multi-cancer detection using an extracellular vesicle protein-based blood test', *Commun. Med.*, vol. 2, no. 1, pp. 1–9, 2022, doi: 10.1038/s43856-022-00088-6.
- [301] J. S. Lane, D. Von Hoff, D. Cridebring, and A. Goel, 'Extracellular Vesicles in Diagnosis and Treatment of Pancreatic Cancer: Current State and Future Perspectives', *Cancers*, vol. 12, no. 6, p. 1530, 2020, doi: 10.3390/cancers12061530.
- [302] Y. Peng and C. M. Croce, 'The role of MicroRNAs in human cancer', *Signal Transduct. Target. Ther.*, vol. 1, no. 1, 2016, doi: 10.1038/sigtrans.2015.4.
- [303] R. Liu *et al.*, 'Serum MicroRNA Expression Profile as a Biomarker in the Diagnosis and Prognosis of Pancreatic Cancer', *Clin. Chem.*, vol. 58, no. 3, pp. 610–618, 2011, doi: 10.1373/clinchem.2011.172767.
- [304] C. Hu *et al.*, 'Potentiality of Exosomal Proteins as Novel Cancer Biomarkers for Liquid Biopsy', *Front. Immunol.*, vol. 13, 2022, doi: 10.3389/fimmu.2022.792046.
- [305] V. Martini, S. Timme-Bronsert, S. Fichtner-Feigl, J. Hoepfner, and B. Kulemann, 'Circulating Tumor Cells in Pancreatic Cancer: Current Perspectives', *Cancers*, vol. 11, no. 11, p. 1659, 2019, doi: 10.3390/cancers11111659.
- [306] G. Kibria *et al.*, 'A rapid, automated surface protein profiling of single circulating exosomes in human blood', *Sci. Rep.*, vol. 6, no. 1, p. 36502, 2016, doi: 10.1038/srep36502.
- [307] Y. Tian *et al.*, 'Protein Profiling and Sizing of Extracellular Vesicles from Colorectal Cancer Patients via Flow Cytometry', *ACS Nano*, vol. 12, no. 1, pp. 671–680, 2018, doi: 10.1021/acsnano.7b07782.
- [308] W. Zhao *et al.*, 'Si nanowire Bio-FET for electrical and label-free detection of cancer cell-derived exosomes', *Microsyst. Nanoeng.*, vol. 8, no. 1, p. 57, Dec. 2022, doi: 10.1038/s41378-022-00387-x.
- [309] D. G. Mathew, P. Beekman, S. G. Lemay, H. Zuilhof, S. Le Gac, and W. G. van der Wiel, 'Electrochemical Detection of Tumor-Derived Extracellular Vesicles on Nanointerdigitated Electrodes', *Nano Lett.*, vol. 20, no. 2, pp. 820–828, 2019, doi: 10.1021/acs.nanolett.9b02741.
- [310] W. Zhang *et al.*, 'Electrochemical micro-aptasensors for exosome detection based on hybridization chain reaction amplification', *Microsyst. Nanoeng.*, vol. 7, no. 1, pp. 1–8, 2021, doi: 10.1038/s41378-021-00293-8.
- [311] X. Ma, Y. Hao, and L. Liu, 'Progress in Nanomaterials-Based Optical and Electrochemical Methods for the Assays of Exosomes', *Int. J. Nanomedicine*, vol. 16, pp. 7575–7608, 2021, doi: 10.2147/IJN.S333969.
- [312] M. Lee *et al.*, 'A pretreatment-free electrical capacitance biosensor for exosome detection in undiluted serum', *Biosens. Bioelectron.*, vol. 199, p. 113872, Mar. 2022, doi: 10.1016/j.bios.2021.113872.

-
- [313] F. Song *et al.*, 'Enrichment-Detection Integrated Exosome Profiling Biosensors Promising for Early Diagnosis of Cancer', *Anal. Chem.*, vol. 93, no. 11, pp. 4697–4706, Mar. 2021, doi: 10.1021/acs.analchem.0c05245.
- [314] J. Li *et al.*, 'Highly Sensitive Exosome Detection for Early Diagnosis of Pancreatic Cancer Using Immunoassay Based on Hierarchical Surface-Enhanced Raman Scattering Substrate', *Small Methods*, vol. 6, no. 6, p. 2200154, Jun. 2022, doi: 10.1002/smt.202200154.
- [315] T.-D. Li *et al.*, 'An ultrasensitive polydopamine bi-functionalized SERS immunoassay for exosome-based diagnosis and classification of pancreatic cancer', *Chem. Sci.*, vol. 9, no. 24, pp. 5372–5382, 2018, doi: 10.1039/C8SC01611A.
- [316] J. M. Lewis, A. D. Vyas, Y. Qiu, K. S. Messer, R. White, and M. J. Heller, 'Integrated Analysis of Exosomal Protein Biomarkers on Alternating Current Electrokinetic Chips Enables Rapid Detection of Pancreatic Cancer in Patient Blood', *ACS Nano*, vol. 12, no. 4, pp. 3311–3320, 2018, doi: 10.1021/acsnano.7b08199.
- [317] T. Kawata *et al.*, 'Improved sensitivity of a graphene FET biosensor using porphyrin linkers', *Jpn. J. Appl. Phys.*, vol. 57, no. 6, p. 065103, Jun. 2018, doi: 10.7567/JJAP.57.065103.
- [318] T. M. Cotton, S. G. Schultz, and R. P. Van Duyne, 'Surface-enhanced resonance Raman scattering from water-soluble porphyrins adsorbed on a silver electrode', *J. Am. Chem. Soc.*, vol. 104, no. 24, pp. 6528–6532, 1982, doi: 10.1021/ja00388a008.
- [319] K. A. Servage, K. Stefanius, H. F. Gray, and K. Orth, 'Proteomic Profiling of Small Extracellular Vesicles Secreted by Human Pancreatic Cancer Cells Implicated in Cellular Transformation', *Sci. Rep.*, vol. 10, no. 1, 2020, doi: 10.1038/s41598-020-64718-6.
- [320] C. F. Ruivo *et al.*, 'Extracellular Vesicles from Pancreatic Cancer Stem Cells Lead an Intratumor Communication Network (EVNet) to fuel tumour progression', *Gut*, p. gutjnl-2021, 2022, doi: 10.1136/gutjnl-2021-324994.
- [321] J. Lötvall *et al.*, 'Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles', *J. Extracell. Vesicles*, vol. 3, no. 1, p. 26913, 2014, doi: 10.3402/jev.v3.26913.
- [322] Z. Andreu and M. Yáñez-Muñoz, 'Tetraspanins in Extracellular Vesicle Formation and Function', *Front. Immunol.*, vol. 5, 2014, doi: 10.3389/fimmu.2014.00442.
- [323] Z. Gao *et al.*, 'Detection of Sub-fM DNA with Target Recycling and Self-Assembly Amplification on Graphene Field-Effect Biosensors', *Nano Lett.*, vol. 18, no. 6, pp. 3509–3515, 2018, doi: 10.1021/acs.nanolett.8b00572.

-
- [324] A. Ganguli, V. Faramarzi, A. Mostafa, M. T. Hwang, S. You, and R. Bashir, 'High Sensitivity Graphene Field Effect Transistor-Based Detection of DNA Amplification', *Adv. Funct. Mater.*, vol. 30, no. 28, p. 2001031, 2020, doi: 10.1002/adfm.202001031.
- [325] M. T. Hwang *et al.*, 'Ultrasensitive detection of nucleic acids using deformed graphene channel field effect biosensors', *Nat. Commun.*, vol. 11, no. 1, 2020, doi: 10.1038/s41467-020-15330-9.
- [326] M. Xue *et al.*, 'Integrated biosensor platform based on graphene transistor arrays for real-time high-accuracy ion sensing', *Nat. Commun.*, vol. 13, no. 1, 2022, doi: 10.1038/s41467-022-32749-4.
- [327] C. Hu *et al.*, 'Potentiality of Exosomal Proteins as Novel Cancer Biomarkers for Liquid Biopsy', *Front. Immunol.*, vol. 13, 2022, doi: 10.3389/fimmu.2022.792046.
- [328] X. Wang, J. Huang, W. Chen, G. Li, Z. Li, and J. Lei, 'The updated role of exosomal proteins in the diagnosis, prognosis, and treatment of cancer', *Exp. Mol. Med.*, vol. 54, no. 9, pp. 1390–1400, 2022, doi: 10.1038/s12276-022-00855-4.
- [329] K. A. Servage, K. Stefanius, H. F. Gray, and K. Orth, 'Proteomic Profiling of Small Extracellular Vesicles Secreted by Human Pancreatic Cancer Cells Implicated in Cellular Transformation', *Sci. Rep.*, vol. 10, no. 1, 2020, doi: 10.1038/s41598-020-64718-6.
- [330] 'NanoFCM -', NanoFCM. [Online]. Available: <https://www.nanofcm.com/>
- [331] J. A. Welsh *et al.*, 'MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments', *J. Extracell. Vesicles*, vol. 9, no. 1, p. 1713526, 2020, doi: 10.1080/20013078.2020.1713526.
- [332] J. Baran *et al.*, 'Circulating tumour-derived microvesicles in plasma of gastric cancer patients', *Cancer Immunol. Immunother.*, vol. 59, no. 6, pp. 841–850, 2009, doi: 10.1007/s00262-009-0808-2.
- [333] D. Xiao *et al.*, 'Combined Exosomal GPC1, CD82, and Serum CA19-9 as Multiplex Targets: A Specific, Sensitive, and Reproducible Detection Panel for the Diagnosis of Pancreatic Cancer', *Mol. Cancer Res.*, vol. 18, no. 2, pp. 300–310, 2020, doi: 10.1158/1541-7786.mcr-19-0588.
- [334] L. Saludas *et al.*, 'Isolation methods of large and small extracellular vesicles derived from cardiovascular progenitors: A comparative study', *Eur. J. Pharm. Biopharm.*, vol. 170, pp. 187–196, 2022, doi: 10.1016/j.ejpb.2021.12.012.
- [335] S. Erlendsson and K. Teilum, 'Binding Revisited—Avidity in Cellular Function and Signaling', *Front. Mol. Biosci.*, vol. 7, 2021, doi: 10.3389/fmolb.2020.615565.
- [336] R. A. Levin *et al.*, 'Development of a reliable assay to measure glypican-1 in plasma and serum reveals circulating glypican-1 as a novel prostate cancer biomarker', *Oncotarget*, vol. 9, no. 32, pp. 22359–22367, 2018, doi: 10.18632/oncotarget.25009.

-
- [337] A. E. Frampton *et al.*, 'Glypican-1 is enriched in circulating-exosomes in pancreatic cancer and correlates with tumor burden', *Oncotarget*, vol. 9, no. 27, pp. 19006–19013, 2018, doi: 10.18632/oncotarget.24873.
- [338] R. E. G. van Hal, J. C. T. Eijkel, and P. Bergveld, 'A general model to describe the electrostatic potential at electrolyte oxide interfaces', *Adv. Colloid Interface Sci.*, vol. 69, no. 1, pp. 31–62, 1996, doi: 10.1016/S0001-8686(96)00307-7.
- [339] P. R. Nair and M. A. Alam, 'Screening-Limited Response of NanoBiosensors', *Nano Lett.*, vol. 8, no. 5, pp. 1281–1285, 2008, doi: 10.1021/nl072593i.
- [340] B. A. Brown *et al.*, 'Charge Detection Mass Spectrometry Measurements of Exosomes and other Extracellular Particles Enriched from Bovine Milk', *Anal. Chem.*, vol. 92, no. 4, pp. 3285–3292, 2020, doi: 10.1021/acs.analchem.9b05173.
- [341] S. S. Kwon *et al.*, 'Electrical Double Layer of Supported Atomically Thin Materials', *Nano Lett.*, vol. 19, no. 7, pp. 4588–4593, Jul. 2019, doi: 10.1021/acs.nanolett.9b01563.
- [342] S. Ramadan *et al.*, 'Carbon-Dot-Enhanced Graphene Field-Effect Transistors for Ultrasensitive Detection of Exosomes', *ACS Appl. Mater. Interfaces*, vol. 13, no. 7, pp. 7854–7864, 2021, doi: 10.1021/acsami.0c18293.
- [343] W. Huang *et al.*, 'Integrated Rayleigh wave streaming-enhanced sensitivity of shear horizontal surface acoustic wave biosensors', *Biosens. Bioelectron.*, vol. 247, p. 115944, Mar. 2024, doi: 10.1016/j.bios.2023.115944.

Appendices

2024/4/10 14:03

RightLink - Your Account

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Apr 10, 2024

This Agreement between Tianyi Yin ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	
License date	Apr 10, 2024
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Nanotechnology
Licensed Content Title	Raman spectroscopy as a versatile tool for studying the properties of graphene
Licensed Content Author	Andrea C. Ferrari et al
Licensed Content Date	Apr 4, 2013
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Would you like a high resolution image with your order?	no
Will you be translating?	no
Circulation/distribution	50000 or greater
Author of this Springer Nature content	no
Title of new work	Graphene field effect transistor biosensor platform for detection of disease biomarkers in physiological solutions
Institution name	Imperial College London
Expected presentation date	Apr 2024
Portions	Figure 1
Requestor Location	Tianyi Yin
	Attn: PhD Thesis
Total	0.00 USD

Terms and Conditions

Springer Nature Customer Service Centre GmbH Terms and Conditions
The following terms and conditions ("Terms and Conditions") together with the terms specified in your [RightLink] constitute the License ("License") between you as Licensee and Springer Nature Customer Service Centre GmbH as Licensor. By clicking 'accept' and completing the transaction for your use of the material ("Licensed Material"), you confirm your acceptance of and obligation to be bound by these Terms and Conditions.

1. Grant and Scope of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, non-sublicensable, revocable, world-wide License to reproduce, distribute, communicate to the public, make available, broadcast, electronically transmit or create derivative works using the Licensed Material for the purpose(s) specified in your RightLink License Details

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Apr 10, 2024

This Agreement between Tianyi Yin ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	
License date	Apr 08, 2024
Licensed Content Publisher	Springer Nature
Licensed Content Publication	NPG Asia Materials
Licensed Content Title	The electronic properties of graphene and carbon nanotubes
Licensed Content Author	Tsuneya Ando
Licensed Content Date	Oct 21, 2009
Type of Use	Thesis/Dissertation
Requester type	academic/university or research institute
Format	print and electronic
Portion	figure/table/illustration
Number of figure/table/illustrations	1
Would you like a high resolution image with your order?	no
Will you be translating?	no
Circulation/distribution	1 - 29
Author of this Springer Nature content	no
Title of new work	Graphene field effect transistor biosensor platform for detection of disease biomarkers in physiological solutions
Institution name	Imperial College London
Expected presentation date	Apr 2024
Portions	Figure 2
Requester Location	Tianyi Yin
	Attn: PhD Thesis
Total	0.00 USD

Terms and Conditions

Springer Nature Customer Service Centre GmbH Terms and Conditions

The following terms and conditions ("Terms and Conditions") together with the terms specified in your [RightLink] constitute the License ("License") between you as Licensee and Springer Nature Customer Service Centre GmbH as Licensor. By clicking 'accept' and completing the transaction for your use of the material ("Licensed Material"), you confirm your acceptance of and obligation to be bound by these Terms and Conditions.

1. Grant and Scope of License

1.1. The Licensor grants you a personal, non-exclusive, non-transferable, non-sublicensable, revocable, world-wide License to reproduce, distribute, communicate to the public, make available, broadcast, electronically transmit or create derivative works using the Licensed Material for the purpose(s) specified in your RightLink License Details.

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Apr 10, 2024

This Agreement between Tianyi Yin ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	
License date	Apr 10, 2024
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Communications
Licensed Content Title	Graphene nanoelectronic heterodyne sensor for rapid and sensitive vapour detection
Licensed Content Author	Girish B. Kulkarni et al
Licensed Content Date	Jul 7, 2014
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	2
Would you like a high resolution image with your order?	no
Will you be translating?	no
Circulation/distribution	50000 or greater
Author of this Springer Nature content	no
Title of new work	Graphene field effect transistor biosensor platform for detection of disease biomarkers in physiological solutions
Institution name	Imperial College London
Expected presentation date	Apr 2024
Portions	Figure 1, Figure 3
Requestor Location	Tianyi Yin
	Attn: PhD Thesis
Total	0.00 USD

Terms and Conditions

Springer Nature Customer Service Centre GmbH Terms and Conditions

The following terms and conditions ("Terms and Conditions") together with the terms specified in your [RightLink] constitute the License ("License") between you as Licensee and Springer Nature Customer Service Centre GmbH as Licensor. By clicking 'accept' and completing the transaction for your use of the material ("Licensed Material"), you confirm your acceptance of and obligation to be bound by these Terms and Conditions.

1. Grant and Scope of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, non-sublicensable, revocable, world-wide License to reproduce, distribute, communicate to the public, make available, broadcast, electronically transmit or create derivative works using the Licensed Material for the purpose(s) specified in your RightLink License Details.

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Apr 10, 2024

This Agreement between Tianyi Yin ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	
License date	Apr 10, 2024
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Biomedical Engineering
Licensed Content Title	Rapid and ultrasensitive electrochemical detection of ions, biomolecules and SARS-CoV-2 RNA in unamplified samples
Licensed Content Author	Liqian Wang et al
Licensed Content Date	Feb 7, 2022
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Would you like a high resolution image with your order?	no
Will you be translating?	no
Circulation/distribution	50000 or greater
Author of this Springer Nature content	no
Title of new work	Graphene field effect transistor biosensor platform for detection of disease biomarkers in physiological solutions
Institution name	Imperial College London
Expected presentation date	Apr 2024
Portions	Figure 2
Requestor Location	Tianyi Yin
	Attn: PhD Thesis
Total	0.00 USD

Terms and Conditions
Springer Nature Customer Service Centre GmbH Terms and Conditions
The following terms and conditions ("Terms and Conditions") together with the terms specified in your [RightLink] constitute the License ("License") between you as Licensee and Springer Nature Customer Service Centre GmbH as Licensor. By clicking 'accept' and completing the transaction for your use of the material ("Licensed Material"), you confirm your acceptance of and obligation to be bound by these Terms and Conditions.

1. Grant and Scope of License
- 1.1. The Licensor grants you a personal, non-exclusive, non-transferable, non-sublicensable, revocable, world-wide License to reproduce, distribute, communicate to the public, make available, broadcast, electronically transmit or

ELSEVIER LICENSE TERMS AND CONDITIONS

Apr 10, 2024

This Agreement between Tianyi Yin ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	
License date	Apr 08, 2024
Licensee Content Publisher	Elsevier
Licensee Content Publication	Materials Today Chemistry
Licensee Content Title	Toward clean and crackless polymer-assisted transfer of CVD-grown graphene and its recent advances in GFET-based biosensors
Licensee Content Author	M. Bahr, B. Shi, K. Djebbi, M. A. Elaguech, D. Zhou, M. Ben Ali, C. TIBI, D. Wang
Licensee Content Date	Dec 1, 2021
Licensee Content Volume	22
Licensee Content Issue	n/a
Licensee Content Pages	1
Start Page	100578
End Page	0
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of new work	Graphene field effect transistor biosensor platform for detection of disease biomarkers in physiological solutions
Institution name	Imperial College London
Expected presentation date	Apr 2024
Portions	Figure 14
Requestor Location	Tianyi Yin
	Alinc PhD Thesis
Publisher Tax ID	GB 494 6272 12
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your RightLink account and that are available at any time at <https://myaccount.copyright.com>).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

<https://100.copyright.com/myAccount/track/view/printableLicenseDetail.aspx?ref=13691da1-5c3b-4764-ba06-c8084c5d6c3b&termId=>

1/4



This is a License Agreement between Tianyi Yin ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder Identified in the order details below. The license consists of the order details, the Marketplace Permissions General Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

All payments must be made in full to CCC in accordance with the Marketplace Permissions General Terms and Conditions below.

Order Date	11-Apr-2024	Type of Use	Republish in a thesis/dissertation
Order License ID	1472328-1	Publisher	AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE
ISSN	0036-8075	Portion	Image/photo/illustration

LICENSED CONTENT

Publication Title	Science	Rightsholder	American Association for the Advancement of Science
Article Title	The biology, function, and biomedical applications of exosomes.	Publication Type	Journal
Date	01/01/1880	Issue	6478
Language	English	Volume	367
Country	United States of America		

REQUEST DETAILS

Portion Type	Image/photo/illustration	Distribution	Worldwide
Number of Images / Photos / Illustrations	1	Translation	Original language of publication
Format (select all that apply)	Print, Electronic	Copies for the Disabled?	No
Who Will Republish the Content?	Author of requested content	Minor Editing Privileges?	No
Duration of Use	Life of current edition	Incidental Promotional Use?	No
Lifetime Unit Quantity	More than 2,000,000	Currency	USD
Rights Requested	Main product		

NEW WORK DETAILS

Title	Graphene field effect transistor biosensor platform for detection of disease biomarkers in physiological solutions	Institution Name	Imperial College London
		Expected Presentation Date	2024-04-10
Instructor Name	Tianyi Yin		

ADDITIONAL DETAILS

Order Reference Number	N/A	The Requesting Person / Organization to Appear on the License	Tianyi Yin
------------------------	-----	---	------------

REQUESTED CONTENT DETAILS