PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVII and XXXVIII cycles)

Name of PhD student: Jesmina Rexha

Title of PhD research: Exploring the Potential of Graphene Field-Effect Transistors in Biosensing for Health and Environment

Name of PhD supervisor: Prof. Daniele Di Marino
Research lab name: Molecular Biology & Computational Biophysics Laboratory

Cycle:
[ ] XXXVI
[X] XXXVII

PhD Curriculum::
[ ] Marine biology and ecology
[X] Biomolecular Sciences
[ ] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[ ] Actea Mobile Laboratory
[ ] Advanced Instrumentation lab
[ ] Aquarium
[ ] MassSpec lab
[X] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate: .....

ABSTRACT (1000 characters, including spaces):

Graphene Field Effect Transistors (gFETs) offer high sensitivity and rapid response, making them versatile for detecting molecules of various sizes, including Extracellular Vesicles (EVs) and environmental pollutants. This technology holds promise for early disease diagnosis and environmental monitoring. In this study, we adapted a gFET biosensor (Fig.1) designed for SARS-CoV-2 detection[1] to track EVs from liquid biopsy samples and detect pollutants using tailored proteins as probes. We successfully isolated and characterized EVs, achieving high specificity and sensitivity in EV detection targeting the CD63 marker. Additionally, we used the gFET biosensor with PPAR-γ protein to detect environmental pollutants. Analysing EVs in liquid biopsy has potential for disease insights, while environmental pollutant detection can transform disease diagnosis, monitoring, and environmental surveillance.

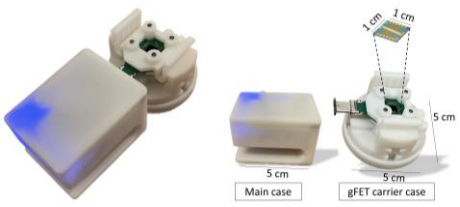


Figure 1. *Photograph of the G-Sense biosensor*

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

- BACKGROUND

Field-Effect Transistors (FETs) are bioanalytical sensors consisting of three key components: a source, a drain, and a gate, separated by a channel. In FET biosensors, the interaction with biological analytes is transduced as a change in the electrical conductance of the sensor [2]. Among the different types of FET, graphene field effect trransistors (gFET) have emerged promising for biosensing due to their unique properties marked by high sensitivity and fast response times[2,3]. gFETs are versatile tool able to detect biologically relevant molecules of different size, from relatively large entities like Extracellular Vesicles (EVs), 150 nm in size, to small molecules as emerging environmental pollutants.

Extracellular Vesicles have emerged as promising disease markers, paving the way for liquid biopsy, a rapid and non-invasive diagnostic approach, thus facilitating early disease detection, enabling timely interventions and ultimately improving patient outcomes. EVs are nanosized vesicles that contain lipids, proteins, and various nucleic acids, including miRNA, lncRNA, and DNA. They are ubiquitously produced by cells throughout the human body and can be detected in numerous bodily fluids, such as blood, urine, saliva, breast milk, cerebrospinal fluid, and synovial fluid. Tumor-derived vesicles can be used as effective surrogate biomarkers to define tumor type and stage, as well as to monitor treatment response[4].

On the other hand, the detection of emerging pollutants is of paramount importance, as these chemicals and compounds have been recently identified as environmental and human health hazards. These pollutants include a variety of compounds such as antibiotics, drugs, steroids, endocrine disruptors, hormones, industrial additives, chemicals, and microplastics which can significantly affect the main physiological functions[5]. Monitoring and mitigating the presence of emerging pollutants is crucial for safeguarding ecosystems and human well-being.

- SCIENTIFIC AIMS

This study aims to utilize a GFET-based biosensor (G-Sense) (Fig.1), originally designed for SARS-CoV-2 detection[1], to track the presence of extracellular vesicles from liquid biopsy and of environmental pollutants in wastewater. By harnessing the versatility of the G-Sense biosensor, we intend to provide a comprehensive analysis tool that can contribute to both medical diagnostics and environmental monitoring, offering valuable insights into disease detection and pollution assessment.

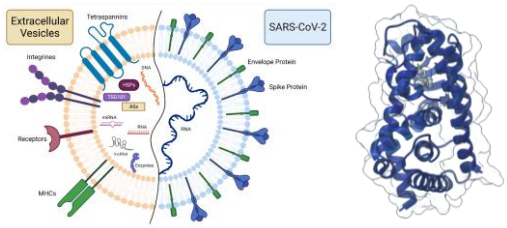


Figure 2. *(A) Picture comparing the biological composition of SARS-CoV-2 and extracellular vesicles. (B) Illustration of PPAR-γ protein (PDB ID 7WGL) used for the detection of environmental pollutants*

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Objective.

To verify the interaction between CD63 marker and antibody anti-CD63, or TIMP1-His or TIMP1-Fc

Methods.

Design of the bioeceptor: An *in silico* characterization through modelling and molecular dynamic simulation was carried out to verify the oligomeric state of the receptor TIMP1. Then, 12% SDS-PAGE gel under reducing and non-reducing conditions was run. 2 ug of TIMP1-His or TIMP1-Fc samples were placed at 100°C under denaturing conditions with Laemmli sample buffer reduced by β-mercaptoethanol or under non-reducing condition using a sample.

Functionalization of the graphene: The graphene layer was modified with the incubation of 5mM 1-pyrenebutanoic acid succinimidyl ester (PBASE) for 2h at R.T. Then, 0.25mg/mL of antibody anti-CD63, TIMP1-His or TIMP1-Fc protein was added and left overnight at 4°C in a humidified environment. Finally, the N-hydroxysuccinimide ester (NHS) free groups of PBASE were passivated with Glycine 100mM.

Electrical Measurements: Electrical Measurements were performed using I_{DS}−V_{GS} curve (Transfer Curves) as electrical metric. Transfer curves were obtained while operating in liquid gating condition, maintaining fixed bias V_{ds} 0.050 V between source and drain electrodes and by sweeping the gate voltage V_g from 0 to

1.5V. The resulting current I_{ds} were plotted as a function of the gate bias. The blank measurements were acquired working in liquid gate condition with 1X PBS pH 7.4. Then, the graphene chip was incubated at R.T. with different concentrations of recombinant CD63 or eIF5A proteins. The Dirac point value which refers to the minimum point with equal population of both positive and negative carriers of the blank and samples were compared using paired t-test implemented in Python.

Expected/Obtained Results.

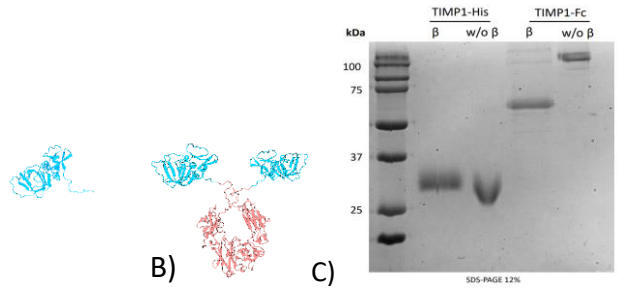


Figure 3. *Picture showing the oligomeric state of the TIMP1 receptor. Molecular simulations reveals that (A) TIMP1-His is a monomer while (B) TIMP-Fc is a dimer. (C) SDS-PAGE under reducing and non-reducing conditions confirmed the in silico results.*

The *in silico* results showed that TIMP1-His is a monomer and maintains this structure over time while the TIMP1-Fc receptor forms a stable dimer and maintains this structure even after 400ns of simulation (Fig. 3A,B). These findings were experimentally confirmed by SDS-PAGE gel electrophoresis both under reducing and non-reducing conditions (Fig.3C). Thus, having a receptor that dimerises enhances its binding to the CD63 dimer, resulting in stronger binding compared to the monomer TIMP1-His or the antibody that only recognizes a single epitope at a time.

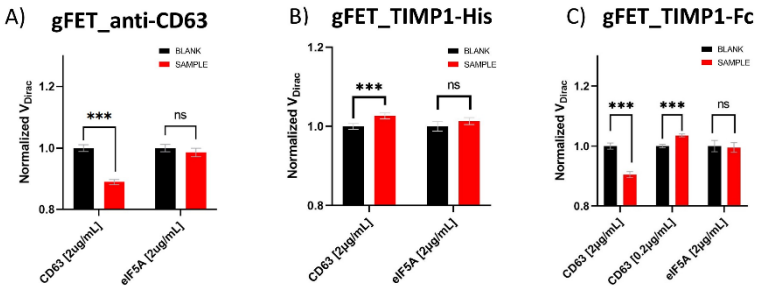


Figure 4*. Comparative bar charts of gFET functionalized with A) antibody anti-CD63 or B) TIMP1-His or C) TIMP1-Fc before (black bars) and after (red bars) the addition of different concentration of samples (i.e., CD63 and eIF5A) *** p < 0.001, error bars represent standard error of the mean (s.e.m.)*

The interaction between CD63 and TIMP1 proteins was assessed through electrical measurements with our biosensor. The graphene layer of the gFET was functionalized with 0.25mg/mL of antibody anti-CD63, TIMP1-His or TIMP1-Fc. Different concentration of the sample (*i.e.*, CD63 or eIF5A recombinant proteins) was added. The specificity was tested with eIF5A recombinant protein which is similar in size to CD63. The biosensor showed specificity and sensitivity in detecting CD63 recombinant protein (Fig.4).

WP 2. Objective.

To isolate and characterize EVs derived from cell lines

Methods.

Ultracentrifugation: The culture media (100mL) of THP1 cell line was collected after 48h of serum-free growth. Cell debris were discarded through a first step of 2000xg centrifugation for 30'. Then the supernatant was subjected to several steps of centrifugations: 9700xg for 30' to pellet apoptotic bodies, cellular debris, and large microvesicles, then at 25000xg for 2h to pellet smaller EVs. The pellet was collected and carefully washed for 2 times with 1 ml of 1X PBS (w/out Calcium and Magnesium) and subjected to centrifugation at 45000xg for 1h. Finally, the EVs pellet was resuspended in 100uL of sterile PBS. All centrifugation steps were performed at 4 °C. The isolated vesicles were stored for a short time at 4°C for quantification and analysis.

Nanotracking Particle Analysis: NTA measurements were performed with a NanoSight LM10 and three videos of either 30 or 60s were recorded of each sample. All measurements were performed at room temperature, never above 25 °C. The software used for capturing and analyzing the data was the NTA 3.3 (Nanosight). Samples containing high particle numbers were diluted before analysis and the relative concentration was then calculated according to the dilution factor.

BCA assay: The bicinchoninic acid (BCA) assay was performed to quantify the total protein amount in the EVs. 2uL or 5uL of isolated vesicles were subjected to BCA with the commercial kit #23235 Thermo Fisher

Scientific. The BCA reagent was diluited following manufacturer instructions and incubated at 60°C x 1h. The samples were added in a 96 MW and read at 570 nm in a plate reader.

FACS cytofluorimeter: 5uL of EVs were incubated with FITC labelled antibody anti-CD63 for 30' and with ExoBright for other 30'. The cytometric analyses were performed by gating events smaller than 1 mm.

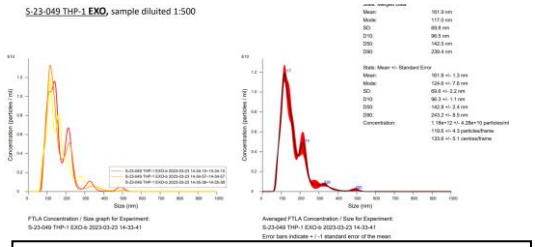


Figure 5. *NTA measurements showing: on the left the 3 measurements performed and on the right the mean ± SD of the*

Expected/Obtained Results.

EVs were successfully isolated through multiple centrifugations steps. The isolated vesicles were quantified through NTA (Fig.5) showing an average size of 117 nm and a concentration of $1.18x10^{12}$particles/ml. The protein content of the undiluted samples was determined by BCA to be 0.1 µg/µl. The positivity to CD63 marker was confirmed by cytofluorimetry.

WP 3. Objective.

To detect isolated EVs

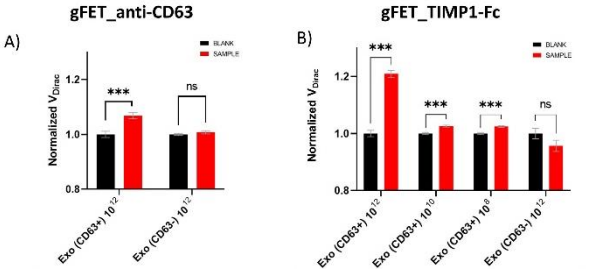


Figure 6. *Comparative bar charts of gFET functionalized with A) antibody anti-CD63 or B) TIMP1-His or C) TIMP1-Fc before (black bars) and after (red bars) the addition of different concentration of samples (i.e., CD63+ EVs, CD63- EVs) *** p < 0.001, error bars represent standard error of the mean (s.e.m.)*

Methods.

The methods used in this WP are the same as in WP1. The same functionalization and electrical measurement protocol were used to detect the isolated vesicles, 20uL of CD63+ or CD63- vesicles.

Expected/Obtained Results.

Different concentration of the isolated EVs were used to perform electrical measurements. The biosensor revealed up to 10^8 EVs/mL (Fig.6).

WP 4. Objective.

To detect environmental pollutants

Methods.

Identification of the bioreceptor: A comprehensive structural-functional study was conducted to identify the receptors that are central in cell functioning and involved in physiological disruption by emerging environmental pollutants.

Electrical measurements: The electrical measurements were carried out following established procedures. An agonist molecule was employed in the electrical measurements at known concentrations.

Expected/Obtained Results.

Structural-functional studies have been conducted on estrogen receptors, aryl hydrocarbon receptors (AHR), and Peroxisome Proliferator-Activated Receptors (PPAR). Among these, PPAR, known for its potential to induce metabolic dysregulation, emerged as a highly suitable candidate for liquid gate measurements due to its excellent solubility. In this context, rosiglitazone, an anti-diabetic drug, has been identified as an agonist and used for electrical measurement. To functionalize the gFET, 0.25 mg/mL of recombinant PPAR-γ was employed. The results demonstrate that our biosensor is not only capable of detecting large molecules but is also sensitive to small molecules.

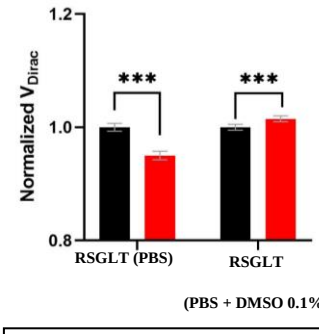


Figure 7. *Comparative bar charts of gFET before (black bars) and after (red bars) the addition of samples. *** p < 0.001, error bars represent standard error of the mean (s.e.m.)*

- REFERENCES

[1] A. Romagnoli, M. D'Agostino, E. Pavoni, C. Ardiccioni, S. Motta, P. Crippa, G. Biagetti, V. Notarstefano, **J. Rexha**, N. Perta, S. Barocci, B. K. Costabile, G. Colasurdo, S. Caucci, D. Mencarelli, C. Turchetti, M. Farina, L. Pierantoni, A. La Teana, R. Al Hadi, F. Cicconardi, M. Chinappi, E. Trucchi, F. Mancia, S. Menzo, B. Morozzo della Rocca, I. D'Annessa, D. Di Marino, *Nano Today* **2023**, *48*, DOI 10.1016/j.nantod.2022.101729.

[2] A. Béraud, M. Sauvage, C. M. Bazán, M. Tie, A. Bencherif, D. Bouilly, *Analyst* **2021**, *146*, DOI 10.1039/D0AN01661F.

[3] **J. Rexha**, N. Perta, A. Roscioni, S. Motta, A. La Teana, L. Maragliano, A. Romagnoli, D. Di Marino, *Advanced Sensor Research* **2023**, DOI 10.1002/adsr.202300053.

[4] H. Shao, H. Im, C. M. Castro, X. Breakefield, R. Weissleder, H. Lee, *Chem Rev* **2018**, *118*, 1917.

[5] T. K. Kasonga, M. A. A. Coetzee, I. Kamika, V. M. Ngole-Jeme, M. N. Benteke Momba, *J Environ Manage* **2021**, *277*, 111485.

Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Seminar by dr. Sonia Silvestri – A shot of science (25.10.2022)

2. School: XXVII School of Pure and Applied Biophysics (06.02.2023 to 10.02.2023)

3. Visiting at prof. Michele Guescini Lab. At University of Urbino Carlo Bo - Isolation and characterization of EVs (21.03.2023 to 24.03.2023)

4. PhD Course of Prof. Andrea Frontini (11.04.2023 to 14.04.2023)

5. Seminar by Steen Larsen "Mithocondria in health and disease" (04.04.2023)

6. Seminar by dr. Alessandra Ritacca – A shot of Science (10.05.2023)

7. PhD Course of prof. Beolchini – Informatica (23/24/26.05.2023)

8. PhD Course of prof. Spinozzi – Informatica (26/29.06.2023)

9. Seminar by dr. Flavia Akemi Nitta Fernandez – A shot of science (20.06.2023)

10. PhD Course of prof. Damiani - Theoric lesson (03/06/24.07.2023)

11. Seminar where I presented my PhD project – A shot of science by Jesmina Rexha (11.07.2023)

12. PhD Week – DiSVA – all the seminars

List of periods spent abroad

/

List of conferences/workshops attended and of contributions eventually presented

1. Poster presentation at 2nd DiSVA-MaSBiC Annual Symposium - Protein structure and function in Biology, Medicine and Nanotechnology

2. Poster presentation at XXVII School of Pure and Applied Biophysics (06.02.2023 to 10.02.2023)

3. Poster presentation @Biophysics at Rome (19-20 April 2023)

4. Poster presentation at 3rd EVIta Symposium (13-15 September 2023)

5. Poster presentation at 3rd MaSBiC Annual Symposium – Advances in Protein Science: Exploring Structure, Function, and Beyond

Part 3. PhD student information on publications

If not yet published, please indicate the publication status (submitted, accepted, in preparation...)

List of publications on international journals

J1. Alice Romagnoli, Paolo Moretti, Mattia D'Agostino, **Jesmina Rexha**, Nunzio Perta, Astra Piccinini, Daniele Di Marino, Francesco Spinozzi, Anna La Teana, Structural–Functional Relationship of the Ribonucleolytic Activity of aIF5A from Sulfolobus solfataricus, *Biomolecules* 2022, 12, 1432. https://doi.org/10.3390/biom12101432

J2. Alice Romagnoli, Mattia D'Agostino, Eleonora Pavoni, Chiara Ardiccioni, Stefano Motta, Paolo Crippa, Giorgio Biagetti, Valentina Notarstefano, **Jesmina Rexha**, Nunzio Perta, Simone Barocci, Brianna K. Costabile, Gabriele Colasurdo, Sara Caucci, Davide Mencarelli, Claudio Turchetti, Marco Farina, Luca Pierantoni, Anna La Teana, Richard Al Hadi, Francesco Cicconardi, Mauro Chinappi, Emiliano Trucchi, Filippo Mancia, Stefano Menzo, Blasco Morozzo della Rocca, Ilda D'Annessa, Daniele Di Marino, SARS-CoV-2 multi-variant rapid detector based on graphene transistor functionalized with an engineered dimeric ACE2 receptor, *Nano Today*, Volume 48, 2023, 101729, ISSN 1748-0132, https://doi.org/10.1016/j.nantod.2022.101729.

J3. **Rexha, J.,** Perta, N., Roscioni, A., Motta, S., La Teana, A., Maragliano, L., Romagnoli, A. and Di Marino, D. (2023), Unlocking the Potential of Field Effect Transistor (FET) Biosensors: A Perspective on Methodological Advances in Computational and Molecular Biology. *Adv. Sensor Res.* 2300053. https://doi.org/10.1002/adsr.202300053

List of publications on conference proceedings

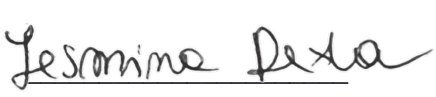
None

List of other publications (books, book chapters, patents)

None

Ancona, 13/10/2023

Student signature



Supervisor signature