PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

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

Name of PhD student: Yessica Roque Diaz

Title of PhD research: Studies of protein aggregation mechanisms: function and dysfunction

Name of PhD supervisor: Dr. Paolo Mariani
Research lab name: Molecular Biophysics

Cycle:
[ ] XXXVII
[x] XXXVIII

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
[x] Advanced Instrumentation lab
[ ] Aquarium
[ ] MassSpec lab
[x] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate:

ABSTRACT (1000 characters, including spaces):

In my first year of PhD, I have mainly worked in three research projects involving macromolecular aggregation, protein-protein, and protein-membrane interaction studies. In this period, and as part of my research, I have gained experience in recombinant protein production and purification, as well as in protein structural characterization by Dynamic Light Scattering (DLS), Small-Angle X-ray and Neutron Scattering (SAXS/SANS) and Atomic Force Microscopy (AFM) techniques. Finally, I have participated in different scientific congress and advanced schools where I have been able of presenting and discussing about my research.

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

- BACKGROUND

Investigation of protein/protein, protein/RNA, and protein/membrane interactions are essential to understand their biological role. The structural characterization of these molecules is a fundamental part of identifying potential molecular targets for developing new therapies.

In my first year of PhD, I have been involved in three research projects that are focused on the study of the mechanism of protein-protein, protein-RNA, and protein-membrane interaction.

My first project focuses on the study of the viral proteins of the SARS-CoV-2 virus. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the ongoing COVID-19 pandemic that has led to the death of millions of people around the globe. Therefore, research on effective antivirals that help reduce the severity of the acute infection is needed [1]. Several viral proteins have been prioritized as SARS-CoV-2 antiviral drug targets due to their role in viral replication, transcription, and recombination during the viral infection. The main protease (Mpro) is considered a promising drug target, as it is dissimilar to human proteases and is highly conserved among the different genera of coronaviruses [2]. Structurally, the active form of SARS-CoV-2 Mpro is a dimer composed of identical subunits, each with a single active site. This makes it possible to achieve inhibition by blocking either the active site of the monomers or the dimerization of the protein. On the other hand, the nucleocapsid (N) protein has gained immense interest as it is structurally associated with the RNA of the coronavirus and therefore represents a potential molecular target [3]. In recent studies, the ability of the N protein to phase-separate in the presence of viral RNA has been reported to play a key role in the viral replication process [4]. However, the mechanism through which this occurs is not yet understood.

My second project focuses on the study of the aggregation of blood-circulating proteins. This project aims to investigate at the structural level the mechanism driving the supramolecular self-assembly of a set of macromolecules involved in blood clotting and that are responsible for significant circulatory diseases. The first protein I am studying is fibrinogen. Fibrinogen is activated into fibrin monomer by thrombin [5], and it has been widely studied because it is considered the final essential building block of the clotting process. However, theoretical polymerization models show a good agreement only for the first steps of the process, while they are not able to describe the mesoscopic scale of the final supra-molecular structure.

Finally, the last project I am focusing on is the study of the Islet Amyloid Polypeptide (IAPP or amylin). Amylin is a neuro-pancreatic hormone that is co-secreted with insulin by pancreatic β cells. The role of amylin is to suppress postprandial glucagon secretion and increase food intake, thus complementing the action of insulin to regulate blood glucose levels. Abnormalities in human amylin folding, secretion, and action have detrimental effects on islet function and glucose regulation by islet amyloidosis and β cell dysfunction in type 2 diabetes (T2D) [6]. In previous works, it has been reported that this peptide interacts with membranes, which have been implicated both as targets of toxicity, via membrane pore formation and/or disruption, and as the catalysts that facilitate peptide aggregation [7]. However, the mechanism through which this occurs remains unclear.

- SCIENTIFIC AIM

I. In the case of SARS-CoV-2 Mpro protein, the goal was to produce the protein with good purity to evaluate the action of potential inhibitors.
   In the case of the SARS-CoV-2 N-protein our goal is to study the structural features of the protein and the mechanism underlying the interaction with the viral RNA to better understand the Nucleocapsid-mediated RNA packaging during the viral replication process. The purpose is to contribute with important structural insights to the development of possible therapies.
II. For my second project, we aim to investigate at the structural level the mechanism driving the supramolecular self-assembly of fibrinogen, induced by thrombin, to understand better the formation of the aggregates, as a function of the kind and the quantity of counterions, to increase the knowledge

of the molecular mechanisms at the basis of this process, and eventually to contribute to the future development of new therapies.

III. Finally, with the study of Amylin polypeptide we intend to determine the influence of the lipid membrane in the formation of the aggregates by studying the interaction of the peptide with model membranes.

- WORKPLAN AND RESEARCH ACTIVITIES

I. Study of Viral proteins of SARS-CoV-2 virus

1.1. Study of the main protease (Mpro)

WP. Production and purification of Mpro

- **Objective.** To obtain the protein with good yield and purity
- **Methods**. To achieve our goal, we produced the Mpro using the expressing vector pGEX-6P-1 (GenScript - clone ID_M16788F), transformed into BL21DE3pLys *Escherichia coli* cells. After the expression, the cells were lysed in lysis buffer and then centrifuged at 5000 rpm for 30 minutes. The supernatant was then purified through affinity chromatography using a Ni-NTA resin. After this, the eluted fraction was dialyzed and concentrated and then further purified using Size Exclusion Chromatography.
- **Expected/Obtained Results**. As a result, we obtained the protein with good purity and a concentration of around 1 mg/ml. This result allowed our collaborators to carry out the inhibition assays to evaluate the action of possible Mpro inhibitors.

1.2. Study of the interaction mechanism between the SARS-CoV-2 Nucleocapsid (N) protein and the viral RNA to better understand the viral replication process.

WP 1. Production and purification of the N-protein

- **Objective.** To obtain the protein with good yield and purity
- **Methods**. To achieve the goal, we purified the recombinant full-length N protein using the expression vector R619-X67-527 (Addgene plasmid # 170204), transformed into BL21DE3pLys *Escherichia coli* cells. After the expression, the cells were lysed in lysis buffer and then centrifuged at 5000 rpm for 30 minutes. The supernatant was then purified through affinity chromatography using a Ni-NTA resin. After this, the eluted fraction was dialyzed and concentrated and then further purified using Size Exclusion Chromatography.
- **Expected/Obtained Results**. As a result, N-protein was obtained with quite good purity and a concentration of about 1 mg/ml, which allowed us to carry out further experiments.

WP 2. Study of the structural characteristics of the N protein and understanding of the mechanistic bases of nucleocapsid-mediated RNA packaging

- **Objective.** Study of the structural features of the N-protein and its interaction with model RNA
- **Methods**. To understand this interaction, we are first studying the structure of the N-protein alone using Small-angle X-ray and Neutron Scattering techniques (SAXS and SANS). After this, we will study the interaction of the N-protein with model RNA of different compositions and lengths to understand at the structural level the origin of the aggregate formation.
- **Expected/Obtained Results**. This study will provide significant structural insights to a better understanding of the replication mechanism of SARS-CoV-2 virus. It will give detailed information on the origin of the interactions between the Nucleocapsid protein and model RNAs (that are expected to change as a function of composition and interaction time), deciphering the role of a merely physical mechanism such as liquid–liquid phase separation in facilitating the viral assembly. Finally, our results will help the design and development of new molecules that could act as inhibitors, making possible the development of more effective antivirals.

II. Study of circulatory blood-clotting proteins

WP. Study of fibrinogen activation into fibrin by thrombin

- **Objective.** To study the structural features of fibrin gel formation in the fibrinogen-thrombin system and the influence of the ionic strength and ions nature in this process.
- **Methods**. The dynamics of thrombin-induced fibrin gel formation have been investigated by DLS. We performed DLS experiment increasing the ionic strength, below and above the physiological value, and modifying the concentration ratio between fibrinogen and thrombin. Thrombin was purchased from Haematologic Technologic Inc, and Fibrinogen from Sigma Aldrich. The last one has been purified by Size Exclusion Chromatography using a Superdex 200 Increase 10/300 GL column to have a precise control of the purity and concentration of the protein. As part of this studies, we have planned to evaluate the rheological properties of the fibrin gel formed under the same experimental conditions mentioned above.

  Expected/Obtained Results. So far, we have confirmed the influence of the thrombin concentration in the formation of fibrin gels: if the concentration of thrombin increases, the velocity of the gel formation is higher. We have also started to evaluate the influence of NaCl concentration in this process: the first result is that as NaCl increases above the physiological value of ionic strength, not only the velocity of fibrin formation gets lower, but different dynamical features appear. Note that the possible correlation with pathological cases could be probably correlated with this basic result, we aim to deeply characterize with other experimental test and techniques. Also, it motivated us to investigate the influence of different ions, and to evaluate the differences in the rheological properties of the fibrin gel in every case, and to obtain morphological information by AFM.

III. Study of Amylin Aggregation

WP. Study of Amylin aggregation using Large Unilamellar Vesicles (LUVs) as membrane model

- **Objective.** To study the influence of the lipid membrane in the formation of Amylin aggregation using LUVs.
- **Methods**. For this study, we are first producing and characterizing the LUVs using different POPC/POPS lipid compositions. LUVs are being produced using the extrusion method, and Dynamic Light Scattering and Confocal Microscopy will be used for its characterization. After this, we will study the structure of the amylin polypeptide in the presence and absence of the LUVs using the Small-angle X-ray Scattering technique to determine the influence of the membrane in the Amylin aggregation process.
- **Expected/Obtained Results**. So far, we have started to produce and characterize the LUVs. As future results, we expect to decipher the role of the biological membrane as an interface that promotes Amylin aggregation.

- REFERENCES

1. El Zowalaty, M.E. and J.D. Järhult, *From SARS to COVID-19: A previously unknown SARS- related coronavirus (SARS-CoV-2) of pandemic potential infecting humans – Call for a One Health approach.* One Health, 2020. **9**: p. 100124.
2. Ullrich, S. and C. Nitsche, *The SARS-CoV-2 main protease as drug target.* Bioorg Med Chem Lett, 2020. **30**(17): p. 127377.
3. Wu, W., et al., *The SARS-CoV-2 nucleocapsid protein: its role in the viral life cycle, structure and functions, and use as a potential target in the development of vaccines and diagnostics.* Virol J, 2023. **20**(1): p. 6.
4. Chen, H., et al., *Liquid–liquid phase separation by SARS-CoV-2 nucleocapsid protein and RNA.* Cell Research, 2020. **30**(12): p. 1143-1145.
5. Kattula, S., J.R. Byrnes, and A.S. Wolberg, *Fibrinogen and Fibrin in Hemostasis and Thrombosis.* Arterioscler Thromb Vasc Biol, 2017. **37**(3): p. e13-e21.
6. Zhang, X.X., et al., *Neuroendocrine hormone amylin in diabetes.* World J Diabetes, 2016. **7**(9): p. 189-97.
7. Cao, P., et al., *Islet amyloid polypeptide toxicity and membrane interactions.* 2013. **110**(48): p. 19279-19284.

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. Design of research: European Projects a.a. 2022/2023
2. Technology transfer and innovation a.a. 2022/2023
3. Introduzione all'ambiente LaTeX per la redazione di documenti scientifici
4. Scuola di Dottorato Nazionale in Heritage Science. Modulo "Tecniche di diffusione a piccolo angolo dei raggi X e dei neutroni per lo studio di materiali nanostrutturati per la conservazione dei Beni Culturali"
5. Giornate Didattiche SISN 2023: Introduzione alle tecniche neutroniche per lo studio microscopico della materia condensata
6. International School of Biophysics "Antonio Borsellino". 48th Course: Memos for biophysics into the future: Lightness, quickness, exactitude, visibility, multiplicity, and consistency

List of periods spent abroad.

1. Small-Angle X-Ray Scattering (SAXS) beamtime at European Synchrotron Radiation Facility (ESRF), Grenoble, France.

List of conferences/workshops attended and of contributions eventually presented.

1. Biophysics@Rome 2023
2. CMD30 - FisMat 2023 International Conference. *Poster Title: "Insights into the mechanism of SARS-CoV-2 main protease inhibitors".*
3. XXXIV Congresso Annuale SISN. *Poster Title: "Purification and SAXS analysis of the Nucleocapsid Protein of the SARS-CoV-2 virus".*
4. Third MaSBiC Symposium: Advances in Protein Science: Exploring Structure, Function, and Beyond. *Poster Title: "Towards understanding the structural features of the SARS-CoV-2 nucleocapsid protein".*
5. International School of Biophysics "Antonio Borsellino". 48th Course: Memos for biophysics into the future: Lightness, quickness, exactitude, visibility, multiplicity, and consistency. *Poster Title: "Towards understanding the structural features of the SARS-CoV-2 nucleocapsid protein"*

Part 3. PhD student information on publications

List of publications on international journals

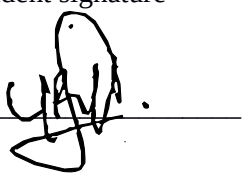
J1. Paciaroni A, Libera V, Ripanti F, Orecchini A, Petrillo C, Francisci D, Schiaroli E, Sabbatini S, Gidari A, Bianconi E, et al. Stabilization of the Dimeric State of SARS-CoV-2 Main Protease by GC376 and Nirmatrelvir. *International Journal of Molecular Sciences*. 2023; 24(7):6062. https://doi.org/10.3390/ijms24076062

List of publications on conference proceedings. (-)

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

[09/10/2023]

Student signature



Supervisor signature