DEPARTMENT OF BIOPHYSICS

Lublin, June 30, 2023

Dr hab. Rafał Luchowski, prof. UMCS

Department of Biophysics, Institute of Physics

University of Maria Curie-Skłodowska

Evaluation of Madhuima Chattopadhyay's doctoral dissertation entitled "The role of biological water in biomimetic cell membrane dynamics and molecular interactions"

Biological membranes are comprehensive structures that form in aqueous environments through the spontaneous organization of lipid molecules. They play a vital role as protective barriers for cells, ensuring their integrity and autonomy. Furthermore, biological membranes play a crucial role in maintaining homeostasis by controlling the microenvironment within cells, which is necessary for proper cellular processes. The complex structure and specific lipid composition of membranes are of significant importance for selective substance transport and intercellular communication, which are essential for the proper functioning of living organisms. Model lipid membranes are commonly used in scientific laboratories to study the functioning of biological membranes under controlled conditions. Information derived from such experiments is particularly valuable to scientific fields such as biophysics, molecular biology, and medicine.

In the context of the mentioned aspects, the doctoral thesis of Ms. Madhuima Chattopadhyay appears as an incredibly interesting and significant study. In her work, Ms. Chattopadhyay focused on investigating the dynamics of lipids depending on hydration states. She expanded then her research to analyze the interactions between lipids, water, and selected ions in the context of the diffusion of such a system. This, in turn, allowed her to gain a deeper understanding of the mechanism of lipid diffusion in different membrane hydration states.

DEPARTMENT OF BIOPHYSICS
Institute of Physics
Faculty of Mathematics, Physics and Computer Science
Maria Curie-Skłodowska University

pl. Marii Curie-Skłodowskiej 1, 20-031 Lublin
tel: (+48 81) 537 62 50
fax: (+48 81) 537 61 91
e-mail: info@biofizyka.umcs.lublin.pl

The doctoral thesis was prepared at the Institute of Physics, Faculty of Materials Engineering and Technical Physics, Poznan University of Technology, in the research group led by the distinguished specialist in microscopy, Dr. hab. Łukasz Piątkowski, a professor at Poznan University of Technology. The institution where the thesis was prepared belongs to the leading centers in the world conducting spectroscopic research on biomimetic membrane systems. The doctoral student gained research experience through participation in three patent applications and numerous projects co-financed by the European Union: the Foundation for Polish Science "FIRST TEAM" (POIR.04.04.00-00-5D32/18-00), the European Molecular Biology Organization (EMBO) Installation Grant (IG 4147), and the EMBO Scientific Exchange Grant (No. 9439), of which she was the principal investigator. She also received two grants as part of the subsidies from her home institution. The results of her studies have been published in three scientific publications and one manuscript in preparation for peer review. These works have high impact factors in the respective journals: Journal of the American Chemical Society (IF 16.383), Biosensors (IF 5.743), and Chemical Science (IF 9.969) - indexed for different years of acceptance for publication. The doctoral student's contribution to these publications is significant, and this conclusion is based on the relevant statements attached to the thesis and the authorship order in the scientific articles. In all of the works, she is the first author and corresponding author. It is also worth mentioning that she is a co-author of two other original research papers that are not included in the submitted thesis.

The evaluated doctoral thesis consists of a compilation of original scientific contributions from the ISI Master Journal List accompanied by an extensive 41-page introduction that includes a list of abbreviations and symbols used. According to formal requirements, the thesis includes abstracts prepared in both Polish and English, as well as a list of cited literature and the author's scientific achievements. The introductory chapters provide an introduction to the topic while clearly defining the objective of the work. The author thoroughly discusses the key issues related to the doctoral thesis topic, focusing on lipid classification and the formation of self-organizing structures by lipids. Furthermore, a mathematical description of molecular diffusion, with particular emphasis on lipid diffusion, is introduced. In the subsequent part of the introduction, the author also discusses the spectroscopic and microscopic techniques employed, which are essential for achieving the planned research tasks and advancing her scientific skills. I would also like to draw attention to the advanced and precise complementary research techniques employed within the doctoral project, including fluorescence microscopy and atomic force microscopy. Mentioning these techniques provides readers with insights into the author's methodology and serves as the basis for further scientific considerations presented in the thesis. In the introductory section of the thesis, the author skillfully acquaints the reader with the challenges faced by contemporary researchers of cell membranes. This ability to incorporate current research problems into the context of the doctoral work demonstrates the author's deep understanding of the subject matter and awareness of existing scientific challenges.

Chapters 7-11 of the evaluated doctoral thesis focus on the detailed results of the research work, which hold significant scientific value. The experimental results presented in these chapters consist of several parts, most of which have already been published. The first part of the results focuses on the characterization of the interaction between the hydration layer and the lipid monolayer. The doctoral candidate's research specifically aimed to determine the influence of the hydration layer on lipid diffusion and intermolecular interactions between the lipids forming the membrane. To achieve these objectives, a method was developed to create biomimetic cell membranes composed of phosphatidylcholine, sphingomyelin, and cholesterol. These membranes allowed for modeling the structure and function of natural cell membranes and the creation of lipid phases with different degrees of ordering (liquid-ordered, L_o, and liquid-disordered, L_d). These different lipid phases were fluorescently labeled with dyes emitting in different spectral regions. Subsequently, a system was constructed to control the humidity conditions of the created membrane by regulating the flow of gas with varying relative humidity. In the initial stage of the research, the doctoral candidate investigated the behavior of the model lipid membranes shortly after their exposure to the external environment. She observed rapid shrinking, folding, and aggregation of the lipid layer, which was caused by rapid water evaporation. Interestingly, these processes did not occur uniformly for both the created self-organizing L_o and L_d phases. The differences arose from their mechanical properties, with the latter undergoing structural changes more rapidly.

The next stage of the analysis was based on the use of dedicated fluorescence microscopy techniques to study the dynamics of molecules. By employing fluorescence recovery after photobleaching (FRAP), diffusion coefficients were determined for the liquid-disordered and -ordered lipid phases at various relative humidity values. Surprisingly, a drastic decrease in lipid diffusion was observed with decreasing humidity only in the range of 90% to 50%. At humidity levels below 50%, diffusion remained at a low, constant level. It was demonstrated that complete hydration forms a water layer around the lipids, which plays a significant role in lipid diffusion through hydrogen bonding. To better understand the moisture-dependent physical mechanisms, experiments were conducted to investigate diffusion coefficients and structural changes of the membrane as a function of temperature. By plotting Arrhenius dependencies, the doctoral candidate discovered that the activation energy for lipid molecule diffusion in dehydrated membranes is approximately twice as high as for fully hydrated systems. The explanation for this phenomenon lies in the fact that water molecules in the first hydration layer protect lipids from electrostatic repulsive interactions. Reducing this water layer leads to increased repulsive interactions between lipids, which in turn increases the activation energy for diffusion. Another category of research presented in the thesis was directly related to temporary and local dehydration occurring, for example, at the interface of two lipid membranes during their fusion process. When two separate lipid layers merge, they displace the hydration layer, requiring the overcoming of a certain hydration force. To investigate this, the doctoral candidate selected two experimental systems: a single-component (lipid) and a three-component (mixture of lipids and cholesterol) system. Using the FRAP technique, she examined the relationship between hydration and lipid mobility. By repeating cycles of dehydration and rehydration, it was determined that the changes in the diffusion coefficient were reversible and reproducible. This led to the conclusion of a strong correlation between two-dimensional lipid diffusion and the availability of water molecules relative to lipids. The correlation between the diffusion coefficient and the degree of hydration of the lipid layer allowed for the precise determination of the local hydration state of biomembranes with an accuracy of about 2-3 water molecules per lipid. Therefore, she proposed a new approach for detecting hydration at the molecular level in biomembranes, using surface lipid diffusion as a measure of local hydration. This discovery is also the subject of a patent application, suggesting that the lipid diffusion coefficient can be considered as a measure of its molecular-level hydration state. Importantly, the methodological approach was not limited to FRAP but also extended to related techniques such as Fluorescence Correlation Spectroscopy (FCS) and Single-Particle Tracking (SPT).

Further research efforts by Ms. Chattopadhyay focused on investigating the behavior of the hydration shell that determines the diffusion rate in the presence of biologically significant alkali metal ions (Na^+, K^+, Mg^{2+}, and Ca^{2+}). She utilized the well-established FRAP technique to determine the surface diffusion coefficients and thereby the dynamics of different lipid phases in the presence of metal ions. She observed that changes in the concentration of Na^+ and K^+ ions have a minimal impact on lipid mobility under fully hydrated conditions. However, the situation changes in the absence of bulk water. In the case of the hydration shell formation around the lipid monolayer, lipid mobility is maintained only in the presence of a specific amount of these metal ions. Interestingly, there is no enhancement of lipid mobility in the presence of Ca^{2+} and Mg^{2+} ions. As an explanation for this situation, Ms. Chattopadhyay suggests that Ca^{2+} ions must bind to more than one oxygen atom of the lipid and displace water molecules in the lipid head region, simultaneously destabilizing the hydration shell. Mg^{2+} ions showed little influence on the measured diffusion coefficient. Through the conducted experiments, the doctoral candidate concluded that alkali metal ions from the first group of the periodic table of elements support mobility under reduced hydration conditions by stabilizing the hydration shell. The strengthening of the hydration shell occurs around the lipid heads. However, in the presence of Mg^{2+} and Ca^{2+} ions, lipid mobility ceases immediately after the removal of bulk water.

I find very interesting the unpublished results by Ms. Chattopadhyay regarding the diffusion of different lipid phases (ordered and disordered) labeled with Atto-633, ASR, and Alexa 488 dyes on a mica surface. Using fluorescence correlation techniques with signal correlation collected from a single measurement point and scanned, she observed that upon removal of bulk water, the mechanism of lipid diffusion transitions from free Brownian motion to anomalous subdiffusion. Ms. Madhuima, using atomic force microscopy, ruled out structural defects in the substrate as the cause of this phenomenon. According to Ms. Chattopadhyay's explanation, the absence of water molecules around the lipid heads accounts for this effect. Under such conditions, electrostatic repulsion can lead to the transient immobilization or complete immobilization part of the lipids. The loss of water molecules causes the lipids to temporarily stiffen, acting as an obstacle for other molecules that are still in motion.

In summary, I would like to emphasize the high editorial quality of the dissertation, including the quality of the presented graphics. I could suggest only minor formal corrections:

- Page 7, the sentence "1-palmitoyl-2-oleoylphosphatidylcholine (POPC) having one unsaturated 16-carbon acyl chain..." should be "1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) having a saturated 16-carbon acyl chain..."
- Page 7, Figure 2.2B contains the label "POPE," while the text refers to "POPC."
- Page 11 and onwards, "The method of preparation of GUVs with alternating current" should be "The method of preparation of GUVs with alternating voltage potential".
- I would suggest adding a space before quotation marks.

The results presented in the dissertation undoubtedly provide interesting information. However, I would like to encourage the doctoral candidate to reflect on the following issues, so that she can express her views based on the experiences gained during the work on the dissertation:

1. Is the phase separation observed by the doctoral candidate truly a result of the mixture of lipids and cholesterol, or could it be an effect of aggregation, for example, of fluorescently labeled proteins (cholera toxin)? What experimental idea does the doctoral candidate have to test and exclude this effect?
2. Present question arises in the context of the information presented in Fig. 1 of the publication with the DOI number: 10.1007/s12013-017-0792-7. According to this publication, a 30% addition of cholesterol is the threshold value between lipid phase separation and the presence of a single phase. What were the reasons for choosing this particular addition in the experiments of the doctoral student?
3. The introduction of gas with varying relative humidity into the sample was presumably done through pipes, and adiabatic expansion occurred in the sample chamber. Was the

sudden temperature factor caused by this expansion taken into account when recording the diffusion coefficient values?

4. The doctoral student observed changes in diffusion coefficients using the FCS technique for different hydration levels. However, this level can influence the refractive index value. Did she take into account the possible change in focal volume caused by the mentioned change in measurement conditions in her measurements?
5. As you are a co-author of papers with Prof. Petra Schwille, I am obliged to ask why the FRAP method was chosen for most diffusion coefficient measurements instead of FCS. Were attempts made, and if so, did the autocorrelation function describing two-dimensional diffusion yield identical diffusion coefficient values? Were the technical conditions mentioned in Section *4.2 Perspectives* of the publication in the journal *Biosensors* so different that obtaining comparable results was impossible?
6. Has the master's student considered the impact of fluorescence labeling on the mobility and potential structural organization of lipids? I would like to share here my personal experimental experience. Certain self-assembled structures of cholesterol and antibiotics that I investigate with my collaborators using AFM disappear in the presence of only 0.05% of labeled cholesterol. I am very interested in your personal opinion and comments on this matter.

In my opinion, the evaluated dissertation meets all the requirements set in doctoral procedures and the conditions specified in the Act of 20 July 2018, Prawo o szkolnictwie wyższym i nauce (Dz. U. 2018 poz. 1668 z późn. zm.). I kindly request the admission of Ms. Madhuima Chattopadhyay to further stages of the doctoral process, especially the public defense.

In summary, I would like to emphasize that Ms. Chattopadhyay has presented a valuable doctoral dissertation based on the results of precisely planned experimental research. These works required a comprehensive knowledge of various methodological approaches. The doctoral dissertation is based on original findings and analyses, which have been published in reputable international specialized journals, as well as three patent applications, which unequivocally demonstrate the exceptional scientific maturity of the doctoral candidate. Ms. Chattopadhyay developed a method for obtaining dehydrated, phase-separated, lipid bilayers by controlling the relative humidity in their environment without the use of external chemical or mechanical modifications. This facilitated her investigation of the structure and dynamics of lipid bilayers over a wide range of hydration states, ranging from a few water molecules per lipid and understanding the behavior of lipids under lower hydration conditions. She also examined the influence of metal ions' presence and their cooperativity with water molecules on lipid diffusion processes. She described the conditions for lipid bilayer fusion at the molecular level. Furthermore, the final chapter of the doctoral dissertation, titled "Perspectives," essentially presents a well-thought-out research plan for future scientific projects. All of this gives the impression that this important topic still has its best moments ahead, and the possible directions for its development suggested by the doctoral candidate appear very promising. Therefore, considering the above, I recognize the significant universal potential of the conducted research, and the high scientific level of the achieved results leads me to perceive the dissertation as exceptional.

Taking into account the relevance of the topic addressed in the preparation of the doctoral dissertation, its scientific level and quality, as well as the scientific and publication activity of the author, I propose to the Discipline Council of Materials Engineering and Technical Physics at Poznan University of Technology to consider the possibility of recognizing the doctoral dissertation as outstanding.

I want to express congratulations to the doctoral candidate and the supervisor on achieving such valuable results. It is a special achievement that highlights the high quality of their research work in the laboratory.