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Doctoral Thesis Review

Review of Emilia Ewa Krok's Doctoral Thesis submitted to the Institute of Physics, Faculty of Materials Engineering and Technical Physics, Poznan University of Technology.

Title of the Doctoral Thesis:

Eukaryotic and prokaryotic biomimetic cell membranes: structure and its relation to environmental conditions

Ph.D. candidate: Emilia Ewa Krok

1. Objective

Cell membranes are key elements in the organization and activities of living cells, which are characterized by inhomogeneous structures. The main and regularly distributed lipidic areas are interrupted by discrete heterogeneities mainly composed of proteins, cholesterol, and other biomolecules. The heterogeneous areas present in these structures are responsible for different vital biological activities; therefore, studying the intricate mechanisms driving the local membrane heterogeneities due to the phase separation in both prokaryotic and eukaryotic cell membrane models is of great importance.

The Thesis mainly focuses on developing membranes with the ability to mimic biological cell membranes and studying the influence of different environmental parameters on the membranes` structure and, in turn, on their functionalities.

Emilia Ewa Krok identified several targets to tackle this complicated scientific problem. The final aim is reached by securing several milestones; indeed, this doctoral study was directed toward: i) evaluating the impact of the environmental pH variation on the formation, dynamics, and stability of phase-separated biomimetic cell membranes; ii) understanding the structural response of the phase-separated supported lipid bilayers (SLBs) to an extensive range of hydration states at the nanoscale; iii) developing binary and ternary lipid mixture-based biomimetic cell membranes with high complexity level. Therefore, the presented study has been designed and oriented in the right direction. New biomimetic membranes have been created, and unexplored behaviors have been successfully investigated in a well-organized methodological way. Additionally, the research activities performed by the candidate during her Ph.D. studies are based on the use of sophisticated instruments applied to develop original methodologies for studying the properties of cell membrane models. The results obtained by Ms. Krok not only give new pieces of scientific information on the mechanism connected to the functionalities of an intricate system as the cell membranes but even open the gate to use that model for novel intriguing discoveries, especially the evaluation of anhydrobiosis in bacterial cell membranes to explore the intimate biochemical paths regulating bacterial survival under dehydration. Overall, the sub-objectives and foremost aim of the Thesis have been completely accomplished.

2. Originality and Results

Biological membranes are essential for cellular function, serving to shield the cell from external fluctuations. Additionally, they play a pivotal role in transporting into and out of the cell of ions and molecules, including amino acids, glucose, and lipids. Indeed, the segregation within membranes and the compartmentalization facilitated by lipid domains are closely associated with numerous biological processes within the body (e.g., protein sorting, regulation of ion channels, signaling pathways, membrane trafficking, cytoskeleton organization, and pathogen entry).

Unfortunately, studying cellular membranes in their natural state poses significant challenges owing to the intricate structural makeup of these systems and the multitude of chemical, biological, and physical phenomena occurring within the cell. Consequently, researchers frequently employ biological cell membrane models, such

as unilamellar vesicles or lipid bilayers.

The applicant developed phantoms exhibiting comparable physical and structural characteristics to native cell membranes (eukaryotic and prokaryotic-like membranes). Most importantly, Mrs. Krok also fabricated structures, which are amenable to modification for targeted investigations into specific biophysical properties, which made it possible to tune the structures and then evaluate the functionalities based on the applied modification, which is an essential step in the field.

All the aforementioned functionalities of cell membranes are affected by specific surrounding conditions, which can impact the organs and organism activities. The candidate studied the influence of three parameters on a scientifically attractive system as supported lipid bilayers (SLBs), which perfectly mimic natural cell membranes. The findings achieved by the applicants were deeply discussed in three different original articles published in high-quality international scientific journals.

In the first published article (Journal of Molecular Liquids), the applicant investigated the behavior of SLB-based cell membranes under varying pH conditions. That Thesis activity was aimed at exploring how phase-separated membranes responded across a broad environmental pH range (from 1.7 to 9.0). A distinct increase in the size of ordered phase domains as pH levels rose, indicating the high sensitivity of lipid membrane structures to pH fluctuations, was observed. Moreover, lipid membranes retained their overall mobility throughout the pH spectrum tested. Domains with specific sizes were formed along the pH variation. Emilia Ewa Krok attributed this phenomenon to variations in the height disparity between lipids comprising liquid-disordered (L_d) and liquid-ordered (L_o) phases under different pH conditions. The results shown are particularly important in the field as they prove the possibility of developing membranes with well-predetermined sizes and shapes of lipid domains without the need for external modifications in composition. That finding opens the way to studying complicated processes like protein binding, signal transduction molecules, and the integration of membrane channels to track transport across membranes in a relatively less complex manner.

In the second presented manuscript (Nanoscale), Mrs. Krok evaluated the nanostructural response of various hydration conditions using phase-separated SLBs as a model. Surprisingly, the SLB structure can tolerate complete desiccation if dehydration occurs gradually and under control without showing defects or perforations.

Furthermore, the applicant noticed that this process induced significant nanostructural rearrangements within the membrane. Indeed, a considerable reduction in hydrophobic mismatch between the L_d and L_o phases appeared during dehydration. Moreover, a key aspect was noticed: this process is entirely reversible.

The last shown publication (BBA - Biomembranes) is playing in the field of biomimetic membranes, but it escapes from the previous specific field (evaluation of the environmental change impact on eukaryotic biomimetic cell membranes). This presented achievement deals with the fabrication and optimization of prokaryotic biomimetic membranes with a gradual rise in their complexity, resembling those found in both gram-positive and gram-negative bacteria. To this end, the applicant employed an electroformation method to generate giant unilamellar vesicles (GUVs) using varying molar ratios of phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (CA). These models were created from binary or ternary lipid blends, allowing precise adjustment of the lipid composition to mimic membranes characteristic of specific bacterial strains (optimizing size, membrane curvature, charge, and lateral organization). Additionally, the applicant discovered that tuning the membrane binding efficiency is possible by modifying the concentration of negatively charged lipids.

The candidate conducted an in-depth investigation of an original and intricate system with meticulousness. The research activities outlined in the Thesis are novel and hold a significant impact within the field of biological membranes. The scientific discoveries merit publication, as they embrace novel approaches and findings. I would like to point out that as a material scientist working on the development of biomaterials, I would be interested in reading a non-original manuscript (e.g., a Review/Perspective/Opinion manuscript) written by Mrs. Krok and her collaborators in this field. Finally, the primary objectives of this Ph.D. Thesis have been the focus of several articles published in highly esteemed international journals by Emilia Ewa Krok, which confirm the completeness and high quality of the doctoral studies carried out by the candidate.

3. Methods

The Ph.D. Thesis submitted by Emilia Ewa Krok contains a comprehensive description of scientific activities, which are entirely experimental-based. The candidate

extensively explored the fabrication and investigation of the structural functionalities of two different biomimetic cell membrane models (both eukaryotic and prokaryotic mimicking structures). In both SLBs- and GUVs-based membranes, the applicant fabricated and studied numerous cases varying the membrane compositions, conducting exhaustive studies to assess the impacts of the environmental parameters on the structural properties and functionalities of the membranes.

The fabrication and optimization of the developed membranes is challenging. Anyway, I would like to point out the high level of complexity of the experimental activities performed by the applicant in characterizing the materials. Most of the techniques used (except for DLS) are time- and effort-consuming. This is particularly true for techniques such as AFM and optical microscopes used in the configuration reported in the presented articles. Frankly, I'm particularly impressed by the quality of the data acquired using AFM and published in the Nanoscale article. Collecting high-resolution AFM topographies of the delicate cell membrane models under controlled humidity is extremely challenging, and I believe the data presented in this article deserves the maximum attention from scientists working in the field.

4. Presentation and Discussion

The Ph.D. Thesis presented by the applicant consists of more than 170 pages and is presented as a cumulative paper Thesis. Three already published articles are the main backbone of the Thesis.

Preceding the articles are an Abstract, a comprehensive Introduction, two sections dedicated to the state-of-the-art in the field, and a section describing the methodology used in detail. Then, after a comprehensive discussion of each publication and connected original texts, the most important results and outlook, as well as scientific achievements and a reference list comprising more than 200 articles, are listed. At the very end of the Thesis, the Declarations of co-authors affirming the applicant's pivotal role in reaching the presented outcomes are reported. All in all, the section arrangement follows the most common one for a Ph.D. Thesis, making this long text reader-friendly.

The Abstract is clear and well-written, moreover, the Abbreviations section is definitely

useful. The Introduction section intends to put the reader in contact with the Thesis scope more than the approached scientific field, which is very useful. Anyway, this section can be improved as I would be glad to see a list of aims and sub-objectives in the" *1.1 Motivation"* part to understand the targets and then have the possibility to compare them with the obtained results highlighted at the end of the Thesis (reported on "*8.1 Summary of results"*).

At this stage, the applicant furnished the main background pieces of information meticulously designing this part in 2 sections (*"2 Cell membrane"* and "*3 Model membrane systems"*), which are both, in turn, structured into three sub-sections. The described notions are essential, moreover, Mrs. Krok was able to skip reporting insignificant pieces of information, avoiding making this part unnecessarily wordy.

Usually, sections like" *4 Experimental techniques"* are not very important because they generally report only well-known pieces of information. Anyway, in this specific case, the presence of this particular Thesis portion is highly appreciated. I envisaged that the applicant spent most of her experimental-dedicated time characterizing the fabricated structure. Moreover, the techniques used are sophisticated, not wholly user-friendly, and have not been used in "standard" configurations, which allows the applicant to pose several innovations from the experimental/technical point of view.

Sections 5, 6, and 7 are focused on summarizing the technical and scientific achievements obtained by the applicant. They are unquestionably the most interesting parts of the Thesis, and the reason is connected to the outstanding results shown. Moreover, I would like to take this occasion to highlight the quality of the scientific results. Indeed, the motivation and assumptions are correct, the experiments have been appropriately set, and finally, the data interpretations and connected discussion are flawless, which makes it challenging to find space for suggesting possible extensive improvements in the reported scientific results.

"8 The most important results of the Thesis and future outlook" is divided into two sub-sections. The structure of the first one can be improved as I believe that the technical findings of each paper can be listed in sections number 5, 6, and 7, respectively; while I would appreciate reading the overall Thesis results here, which of course are very connected to the articles` results, anyway are not obvious identical. On the other hand, the part solely dedicated to outlooks is hugely welcome. This text part shows that Emilia

Ewa Krok not only acquired technical skills but also developed a well-structured scientific approach/mind, which is the intrinsic objective of a Ph.D. pathway.

The last main section section of the Thesis *("9 Scientific achievements"*) is directed toward reporting the technical goals reached by the applicant during her doctoral studies. From that point of view, I should highlight that the reported achievements are outstanding in each of the ten listed sub-sections. Moreover, I'm delighted to see that Mrs. Krok, has already been able to collect public funds (NCN Preludiulm) for developing her scientific idea, confirming that she is a young but at the same time mature scientist with a prospective career.

The Thesis is written in English. In general, the writing style is of high quality, and the text does not contain any significant grammar errors. Given the large volume of material covered and the range of issues addressed, the candidate has accomplished commendable work in maintaining a consistent style. The overall assessment of the Thesis's format and organization is extremely positive.

From the scientific point of view, I would like to remind the candidate of the importance of studying the membrane properties as they are vital building blocks of biological drug carriers for advanced drug delivery systems (e.g., targeted RNA delivery), which can be a further outlook for her scientific path. Additionally, I would like to stress a point that could be viewed as trivial, but that shows a lot about the maturity of Emilia Ewa Krok. We are living in a world dominated by images, and science perfectly reflects it; the fact that the applicant spent time and effort in organizing a Cover Image for her Thesis confirms that Mrs. Krok developed a mature and modern scientific mind, which is favorable for her career as well as for the entire scientific community.

5. Conclusion

My overall assessment of the Thesis is highly favorable. The candidate adeptly tackled one of the most complex challenges in the field. Additionally, Emilia Ewa Krok thoroughly discussed scientific achievements, ensured the consistency of approaches, and showcased the applicability of their original methodology (e.g., AFM topographies of the delicate cell membrane models under controlled humidity) on promising but challenging systems as the investigated SLBs- and GUVs-based membranes

mimicking both eukaryotic and prokaryotic cell membranes, respectively. The presented results are of significant value for advancing the scientific community's knowledge in that specific area. Mrs. Krok tackled challenging scientific questions with an excellent approach and showed a high scientific maturity. Furthermore, the outstanding results achieved by the applicant warrant the utmost attention. For these reasons, I propose allowing her to defend her doctoral Thesis at a public dissertation and considering her for distinction.

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