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Wrocław, 20.07.2023

Review of the dissertation by Madhurima Chattopadhyay entitled: "The Role of Biological Water in Biomimetic Cell Membrane Dynamics and Molecular Interactions" performed at the Poznan University of Technology (PUT), Faculty of Materials Engineering and Technical Physics, Institute of Physics under the supervision of Dr. hab. Eng. Łukasz Piątkowski, Prof. PUT

Cellular membranes act not only as dynamic barriers delineating different biological compartments, but also serve as multifunctional hubs for a wide range of physiological processes. The amphipathic nature of membrane lipids drives them to self-assemble into bilayers, which in turn harbor integral proteins. The overall structure is not only formed due to the hydrophobic effect, but also stabilized by the hydration shell. Thus, water molecules play a central role in the organization and dynamics of membranes and all their components. On the other hand, the dynamics characteristic for all living systems is often linked to substantial reorganization of membrane structures. Physiological processes, such as macromolecular interactions, exocytosis, cell fusion and viral entry, to name a few, require the bilayer structure to be disturbed to initiate membrane fusion, which is directly linked to local and transient dehydration of the membrane. It is the diffusivity of membrane components that is the primary descriptor of these as well as other cellular functionalities. Understanding dynamic behavior of membrane components at reduced water availability will allow to better understand processes linked to membrane transformations at a molecular level.

In her dissertation, Madhurima Chattopadhyay attempts to resolve some of the most important problems in the above-noted area of biology. Specifically, the authoress addressed the question how the diffusion of lipids forming a bilayer is influenced by the degree of its hydration and what is the contribution of physiologically relevant cations in this phenomenon.

For a long time, the answers to these questions remained beyond the grasp of the scientific community, despite being one of the most important issues in modern biology.

The layout of the dissertation follows the best standards for scientific reports and includes two main parts. This is preceded by a brief introduction outlining the general motivation behind the whole project. In the first part of the dissertation, the authoress provides a theoretical background to the reader, where she describes in a concise manner the general knowledge in the field, starting with lipid bilayers as the core of cellular membranes, and discussing biomimetic membrane systems as well as lipid diffusion and lipid-water interactions. Some basic concepts of experimental techniques used within the project were also included. This part is balanced, focused, and sufficient to understand the work performed within the PhD project. However, there are a few issues that would require clarification/correcton. First of all, while the figure 2.2 could be considered as informative, it does not represent sphingosine moiety (which should include an unsaturated hydrocarbon chain) correctly and uses wrong color code for POPE head group. Additionally, the text contains some minor oversimplifications, as lipid droplets are usually something more than micelles (p.6), not all saturated lipids with short acyl chains (e.g. dilauroyl DLPC/DLPG) stay in gel phase at room temperature (p. 9), and biological membrane are not two-dimensional structures (this is not only a matter of molecular dimensions but would also exclude a possibility of flip-flop diffusion depicted in Fig. 4.1). There are also some minor typographical errors (e.g. L_o instead of L_d on p. 36, Van der Walls interactions on p. 37, etc.), but they do not affect the generally positive impression of this chapter.

The second part is a pivotal part of the dissertation since it contains the results obtained from the research. The authoress shows here three original, peer-reviewed research articles published in high impact internationally recognized journals. In all these articles the PhD candidate is simultaneously first and corresponding author. This fact, together with attached declarations of the co-authors, confirms her major contribution to these works. In addition to full-length published articles (together with supplementary information), the dissertation contains a short summary of each item. Such structure helps to better understand not only the basic concepts behind the studies but also individual contribution of the authoress. In an additional chapter, an unpublished data was presented. It is worth noting, that the description of the materials and methods used by the authoress, which gives an overview of all reagents, tools and methodological details of the study, has been done very carefully and the reader will find all the details needed to reproduce the experiments.

The work published in the Journal of the American Chemical Society is based on an innovative experimental setup enabling preservation of supported bilayers (SLBs) at reduced humidity without any additional chemical or physical modifications and rehydrating the system in a fully repeatable way. This, together with standard fluorescence recovery after photobleaching (FRAP) approach was used to explore hydration-dependent diffusivity within supported lipid bilayers, which led to a very interesting observation. While the lipid domain structure of phase separated SLBs was intact in all experimental conditions tested, lipid mobility was drastically affected by dehydration while diffusion activation energy increased approx. 2-fold. Quantitation of the number of water molecules per lipid at various degrees of bilayer dehydration is crucial for understanding multiple biological processes at molecular level. This idea was further extended within the next publication which appeared in the journal Biosensors. Here, the authoress and collaborators used similar experimental setup to study single component SLBs and obtained compatible results. Keeping in mind that real biological systems are not only water and lipids, the PhD candidate took a one step further and included in her system some biologically-relevant ions, such as Na^+, K^+, Mg^{2+} and Ca^{2+}. While describing the obtained results within the paper published in the journal Chemical Science, she concluded that the ion-specific relationship between lipid mobility and bilayer hydration is a consequence of the interplay between lipid binding affinity and the hydration energy of the ions. Since all the above-described conclusions regarding lipid diffusivity were based on FRAP experiments that does not allow to recognize anomalous sub-diffusion, the ultimate challenge of the PhD project was to employ an approach that is based not only on ensemble averaged measurements, but also could provide single-molecule sensitivity. This was accomplished by using two variants of fluorescence correlation spectroscopy (point FCS and scanning FCS). Although the presented results should be considered as preliminary, they strongly suggest that removal of bulk water results in alteration of the lipid diffusion in the phase-separated SLBs towards anomalous sub-diffusion instead of free Brownian motion. This part of the dissertation ends with a brief but fascinating chapter entitled "Conclusions and outlook", where the authoress summarizes her major achievements in light of further studies that would be most interesting to undertake in the direction she pointed out.

While going across the whole material collected within this dissertation it becomes obvious that it consists not only of high-quality results, but the latter are also correctly interpreted and thoroughly discussed in light of previously published reports. On top of that, some very attractive hypotheses on lipid bilayer organization at molecular level were formulated. Nevertheless, there are a few issues listed below that should be addressed in more details by the authoress.

1. First of all, the influence of the support on the properties of the studied bilayers should be a bit more thoroughly discussed. It has been already debated that both the process of membrane deposition and the physical properties of the SLBs strongly depend on ionic interactions between lipids and the support, such as the calcium-mediated interactions with the mica support. SLB-promoting effect of calcium ions is not restricted to negatively charged lipids (such as phosphatidylserine) but extends also to zwitterionic phosphatidylcholines, although in the latter case the effect was considerably less pronounced (see Richter & Brisson 2005 Biophys J 88: 3422, ref [37]). The preliminary results shown on Fig. 11.1 indicate that the lipid diffusion in the lower leaflet does not increase back to its initial value after rehydration. Thus, the question is whether it is only an issue of permanent removal of the water layer of between mica and the lower lipid leaflet of the SLB and whether residual calcium ions may play a role in that phenomenon.

2. Did the authoress consider employing alternative approaches towards SLB formation (also on various supports) in order to eliminate any possible artefacts? For example, deposition of a lower leaflet by the Langmuir–Blodgett technique and the subsequent transfer of an upper leaflet by horizontal dipping the support to another monolayer in a Langmuir trough are possible (e.g. Tamm & McConnell 1985 Biophys J 47: 105, ref [36]). Generally, SLBs prepared by such techniques may have fewer topological defects than those formed by vesicle fusion. This issue could be of critical significance in light of lipid sub-diffusion measurements. On top of that, LB and LS deposition allow to control and adjust the surface pressure and molecular packing area of the SLB (e.g. Kurniawan et al. 2018 Langmuir 34: 15622 ref [43]).

3. The authoress correctly pointed out that while considering diffusion measurements with single-molecule sensitivity STED-FCS (stimulated emission depletion FCS) is an attractive alternative. This has already been successfully used to characterize anomalous sub-diffusion in membranes. However, STED-FCS requires highly stable fluorescent dyes and the incomplete suppression of fluorescence in the STED process challenges the analysis of the auto-correlation curves in FCS (although recently a new fitting model has been developed to overcome this, see Wang et al. 2018 Methods 140-141: 212). On the other hand, another spot

variation FCS approach based on classical confocal microscopy could be implemented (e.g. Mailfert et al. 2020 J Vis Exp. 165). Although it is not as challenging in terms of photophysical properties of the dyes used, important limitation of such svFCS approach results from the classical optical diffraction limit (~200 nm).

There are also some further minor issues which deserve consideration in light of the results presented in the dissertation.

1. As some of the studied SLB systems were based on binary and ternary lipid mixtures containing sphingomyelin (SM) it would be beneficial to discuss a bit more (based on the scheme shown on Fig. 4 of the JACS paper) how changes of the hydration shell would look like in case of SM and how lipid packing would influence it.

2. In the chapter 6.2 some environment-sensitive membrane fluorescent probes were described. Does it reflects further considerations of the authoress towards use of such probes in further experiments? If yes, I would suggest employing fluorescence lifetime measurements for such fluorophores, as such approach would enable getting much higher resolution and dynamic range than observations of shifts of fluorescence spectra (see e.g. Owen et al. 2006 Biophys J 90: L80)

3. It is a bit unclear why in the FCS experiments Alexa 488 dye was used for calibration of the confocal volume and the structural parameter, while the measurements were done with red-light excited membrane fluorophores? It could be expected that the size of the FCS detection volume was considerably smaller for excitation at 488 nm than for excitation at 633 nm. Thus, another dye of known diffusion coefficient, such as Atto 655 (Atto-Tec), D = 426 $mm^2 s^{-1}$ (Garcia-Saez & Schwille 2008 Methods 46: 116), would be a better choice.

4. The language of the dissertation is in general very precise and objectively correct. One issue worth underlining is that lipid vesicles of any kind are in a form of suspension but not solution in aqueous environment.

All in all, the dissertation is of excellent quality and meets all the criteria for doctoral dissertations specified in Art. 187 of the Act of July 20, 2018 "Law on Higher Education and Science" (Journal of Laws of 2018, item 1668, as amended). It proves that Madhurima Chattopadhyay is able to conduct independent scientific work and reflects her outstanding skills in designing experiments, interpreting data, and presenting results in the context of recent scientific reports. The results obtained are of fundamental importance in the field of biophysics of lipid membranes and shed new light on biological processes that require transient and local dehydration of lipid bilayers. It is worth underlining that vast majority of the presented data were already published in prestigious scientific journals. Therefore, I strongly recommend the Doctorate Comission and Material Engineering Discipline Council of the Poznan University of Technology to accept the dissertation of Madhurima Chattopadhyay and distinguish it with an appropriate award.

Yours faithfully,

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Wniosek o wyróżnienie rozprawy doktorskiej autorstwa Madhurima Chattopadhyay zatytułowanej "The Role of Biological Water in Biomimetic Cell Membrane Dynamics and Molecular Interactions"

Wymieniona powyżej praca doktorska składa się w głównej mierze z trzech publikacji w bardzo dobrych czasopismach naukowych o światowej renomie (o łącznym współczynniku oddziaływania IF przekraczającym 28), czym istotnie wyróżnia się spośród innych prac doktorskich. Całość pracy została bardzo starannie przygotowana i opatrzona szerokim komentarzem i wnikliwą dyskusją. Całokształt dorobku naukowego doktorantki oraz jej aktywność w sferze podnoszenia swoich kwalifikacji oraz propagowania nauki świetnie współgra z obrazem młodej, ambitnej i ponadprzeciętnie uzdolnionej naukowczyni. Biorąc pod uwagę wspomniany już w mojej recenzji wybitny wkład w rozwój dziedziny nauki oraz bardzo wysoki poziom merytoryczny całej pracy doktorskiej wnioskuję o jej wyróżnienie stosowną nagrodą.

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