Opponent's review

PhD thesis of Mgr. Alena Koukalová

"Lipid Membranes at the Nanoscale: Single-Molecule Fluorescence Approach"

The submitted Ph.D. thesis focuses on three specific questions related to structure and biological role of lipid membranes. The first part studies the interaction and pore formation of a novel polyene macrolide DDHR with model lipid membranes of different composition and compares it with membrane interactions of structurally related molecules Filipin III and Amphoterisin B. The second part focuses on a still hot topic of lipid membrane biophysics – detection and characterization of microheterogeneities in lateral membrane organization in model membranes, that have relevance to cellular plasma membranes. The last part brings in a topic of membrane fusion and reveals some of molecular mechanisms important for SNARE-mimicking lipopeptides induced membrane fusion. Apart from the lipid membrane motif these topics share also the methodology aspects, namely the advanced fluorescence methods, especially FCS, FLIM and FRET, applied on Giant Unilamelar Vesicles (GUVs), which were used to answer the postulated questions.

The thesis is a collection of printed, submitted (already accepted) and in preparation reviewed scientific publications. The impact factors of journals the publications appeared in is above the average for the research field (Q1 and Q2, IF 7.23, 4.65, 4.12 and 3.44). The papers have been already cited for 28 times. The collection is complemented by theoretical introductions into the field of lipid biomembranes and into fluorescence-based methods used within the thesis, and by research aims of the studies, main results, conclusions and a list of references. I found only minor issues in the introduction into fluorescence techniques, which I address in my questions. I appreciate the clear indication of contributions of the author of the PhD thesis for each of the publications. The language of the thesis is easy to read and understand. In my opinion, it is very good for an author who is not a native English speaker.

The experimental methods used in the studies are complex and the author has shown a good understanding of their principles and the ability to correctly interpret the data. As all the postulated questions were addressed and the robust conclusions obtained on model systems reasonably related to real biological situations, I have to conclude that this thesis has demonstrated the author's ability of independent high-quality scientific work and that Alena Koukalová deserves to obtain her Ph. D. title.

For my own scientific curiosity I would like to ask the author the following questions:

1. In the introduction on fluorescence methods you mention that photophysical properties of FPs are less favourable than those of organic dyes. Which of the properties are especially important for the methods used in the thesis? Also, in some lipid membrane studies researchers are using quantum dots. How do they compare to FPs and organic dyes? Could you use QDs for FRET studies?
2. In the introduction there are some statements that might be slightly misleading for a non-experienced reader. Could I ask for a short clarification on what exactly was meant by:
   - fluorescent proteins are … too bulky to serve as appropriate fluorophores for labelling lipids – can FP label lipids at all? - page 30 of pdf file
   - Confocal improves resolution – page 32 of pdf file
   - two-photon excitation minimizes photodamage – page 33 of pdf file

- Sufficient amount of photons for TCSPC and FLIM - pages 36 and 37 of pdf file
3. For FCS you mention, without a citation, that the optimum dye concentration is one molecule per detection volume. Is that correct in all cases, even in case when the diffusion is slow compared to acquisition time?
4. In the leakage essay, can the net charge and/or hydrophobicity of the leaking molecules (fluorescence dyes) play a role for the data interpretation?
5. What are biologically relevant DDHR concentrations? Are they similar to test experiments?
6. Your data suggest that DDHR prefers lipid ordered phases. Could it be potentially used as a Lo marker after modification by attachment of fluorescent dye?
7. Images of GUVs suggest that there is quite strong photoselection of dye molecules oriented parallel to the polarization axis of linearly polarized laser line. Can this, or in general all polarization related effects, influence the results obtained by FRET?
8. DiD molecule is known to have light-induced cis-trans isomerization with almost equal distribution of both states. One of the states is bright and one dark. Can this influence FRET results and if yes, was that implemented in MC simulations?
9. Some studies suggest that lipid tracer DiD shows significantly slower diffusion compared to head-labelled lipid analogues in supported lipid bilayers. Have you observed anything similar?
10. Your group has published several papers combining fluorescence methods with molecular dynamics simulations. How close (or far away) are you to model the nano-heterogeneities described in section two?
11. Would the FRET approach for detecting microheterogeneities be applicable for living cells? For example in combination with fluorescence under cryo-conditions to fix the cells for prolong data acquisition.
12. Have you considered using extended FCS methods, namely FLCS or FSCS, combined with dyes sensitive to microenvironment, to follow the dynamic exchange of these molecules between different lipid phases in your model systems? Similar to Nicovich et. al. "FSCS Reveals the Complexity of Lipid Domain Dynamics in the Plasma Membrane of Live Cells", Biophys. J. 2018?
13. In the third section you use FCCS method to quantify the interaction of different molecules. You conclude that in the positive case some of the molecules remain free even at saturation by its binding partner because the amplitude of the cross-correlation function does not equal the amplitude of the respective autocorrelation function. Have you tried some positive doubly-labelled control to rule out the fact that the lower cross-correlation amplitude is not caused by labelling efficiency, photobleaching, or other effects?
14. Was there any difference for the photon-antibunching experimental setup than for FCS? The used laser power, a longer acquisition time, a pinhole size? As usually AB requires more photons for reliable statistics, are GUVs stable enough for the extended, usually tens of minutes, measurement?

In Vestec

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