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Review of PhD thesis of Dusan Mrdenovic entitled "Amyloid β interaction with model cell membranes – insight into the mechanism of the Alzheimer's disease etiology and a novel therapeutic approach"

The PhD thesis of Dusan Mrdenovic entitled "Amyloid β interaction with model cell membranes – insight into the mechanism of the Alzheimer's disease etiology and a novel therapeutic approach" was prepared under supervision of Prof. Włodzimierz Kutner, and Dr. Piotr Pięta was the auxiliary supervisor (both from Institute of Physical Chemistry, Polish Academy of Sciences). The research was carried out in the framework of International Doctoral Studies in Chemistry at the Institute of Physical Chemistry of the Polish Academy of Sciences, in collaboration with Prof. Jacek Lipkowski (University of Guelph, Canada). There are 3 papers included in the thesis; 2 of them are already published in Langmuir and Nanoscale Advances and 3 submitted but not yet published. Together with the papers that are beyond the scope of the thesis, Mr. Dusan Mrdenovic is co-author of 4 published papers and 3 submitted in the years 2019-2020.

The thesis contains 164 pages and consists of 6 Chapters, first of them devoted to basic knowledge and description of the state of the art in the fields of Alzheimer's disease, lipid membranes, and amyloid β formation and aggregation. In Chapter 2, materials, procedures and the methods used are described. Chapters 3 to 5 are devoted to the experimental results and their discussions, and each of them has the introduction and conclusions parts. Chapter 6 contains the research summary and prospects for this line of research and in the end 374 literature references are listed.

In the Abstract preceding the Introduction, the Author explains the structure of the dissertation, and defines the main objectives – identification of the toxic forms of amyloid β and elucidation of the mechanism of membrane permeation by the oligomers of amyloid β. An additional part helping the reader to understand the content is the list of all abbreviations.

In the Introduction (Chapter 1), a hypothesis of amyloid cascade is presented. It is suggested that the toxicity of amyloid fibrils and monomers is lower than that of oligomers which are highly toxic.

The structural aspects and differences in the lipid planar and non-planar nanostructures are explained, and both simple bilayer systems and rafts appearing in the biomembranes are considered, also in relation to the Alzheimer disease. Examples of simple model systems, e.g. black lipid membranes, various supported lipid bilayers are also mentioned including those with a hydrophilic spacer between the lipid bilayer and the solid substrate. The latter allows water molecules to be on both sides of the lipid bilayer as in the case of a biological membrane. The differences between the behavior of amyloids of different lengths $A\beta_{42}$Os and $A\beta_{40}$Os in contact with model membranes are also analyzed. One should perhaps include also other mechanisms of aggregation as those based on the interactions with metal ions.

The relevance of studies performed using simple e.g. DOPC layers is often questioned. Therefore, the lipid extract or the POPC/Chol/SM/GM1 mixture chosen by the Author to prepare the vesicles are certainly a better approach to prepare a biomimetic membrane.

The review of different hypotheses, well presented in this part of the PhD thesis, clearly shows how complex is the subject of this PhD study. Maybe this is why, in Chapter 1.4, the Author returns to more detailed explanations of the objectives of the work which were already introduced in the Abstract.

In Chapter 2, Mr. Dusan Mrdenovic describes the supported bilayer preparation via vesicle fusion and presents the methods used for monitoring of the lipid film properties and topology. A more detailed description of the mechanism of the lipid vesicles spreading and bilayer formation on Au surfaces was reported by Pawlowski et al (Langmuir, 2015), and citing this mechanism would improve this really short although important chapter.

The methods used in the investigations are described clearly and the part devoted to electrochemical impedance spectroscopy is really educational. It combines in one chapter most important recent reports concerning EIS membrane studies and includes Bode plots analysis of homogenous and defective layers on electrodes. The description of IR and especially PM-IRRAS is also well prepared and shows the utility of these methods for the studies of lipid molecules orientation and their conformation changes in the supported lipid bilayers.

Starting from Chapter 3, the results of the investigations are presented. They are organized as discussions of separate papers in chronological order. Such arrangement: each chapter with Abstract, Experimental, Results and discussion and Conclusions parts causes a series of unnecessary repetitions and overlapping information. For example, the peptide

preparation is repeated three times, and the texts on pages 70/71, 90/91 and 116/117 are almost identical.

In Chapter 3, the Author describes the interactions of the amyloid oligomers with the lipid membrane composed of the brain lipid extract. The composition of the total lipid extract is not described, although it would be interesting to know what are the components of the membrane. Usually such extracts are not very stable, therefore, the obtained vesicles can be not reproducible. More comment on this issue would be helpful.

It surprised me that in the first paper, the bilayers are formed by dropping a 40 microliter vesicle sample on mica and leaving it for 45min instead of the common approach of contacting the mica surface with a solution containing vesicles for a long time. Is multilayer deposition not favored in such an approach? Another question concerns sonication of the peptide solution with the lipid vesicles for 10min only. Does it provide enough time for partitioning and establishing an equilibrium between the peptide solution and the curved lipid layer? Usually longer times are needed, and still the actual ratio in the membrane is different from the ratio of peptide to lipid assumed in the experiment. Thinning of the bilayer due to DMSO penetration cannot be avoided, and during fusion the peptide may change its location in the flat bilayer compared with that in the highly curved vesicle. More comments on such uncertainties concerning the composition and stability of the bilayer would be useful.

The AFM topography images of the monomers and oligomers of different sizes and shapes on the mica surface are described in a detailed way. They allowed to establish the time dependence of the aggregation process. The images of fibrils formed at the long time-scales are of very good quality. The case of fused vesicles seems to be much more complicated and interpretation of the images was really difficult. This was due in part to changes of the conformation and organization of the phospholipid components in the layer which accompany the oligomer permeation. The Author draws attention to all difficulties and uncertainties of the measurements proving his careful work during the experiments and data elaboration. As a result of this experimental work, the graduate student has gained significant expertise. The results confirm that spreading of vesicles is influenced by the presence of small oligomers and both extraction and complex formation with lipids takes place. I am not fully convinced that unfused vesicles or half-spheres are not present in the images e.g. in Fig. 3.12. Anyway, the time resolved imaging convinces of the pore formation by the larger oligomers. The interaction mode of the small oligomers becomes more clear for me when the supported bilayer is simply exposed to a solution containing the small oligomers for different length of time as shown in Figs 3.16 and compared with 3.17, where no oligomers are present in the PBS solution. Probably any

lipid extraction processes involved could be detected by quartz microbalance measurements. The results presented in this paper show the differences between the behavior of larger and smaller size oligomers in contact with the bilayer.

In Chapter 4, the Author focused on the lipids forming the cell membrane and changes of electric properties of the membrane under influence of amyloid β. The methods used are different; instead of AFM the IR spectroscopy and electrochemical methods are employed. Since the monomers did not affect the membrane structure, mainly oligomers are considered. Here, the vesicles consisted of a mixture of DSPE, DPPC, cholesterol, SM and GM1 in a ratio chosen to mimic the rafts formed in the brain membranes of AD patients reported by Martin et al. The vesicles were mixed with the amyloid to achieve a certain ratio but it was not explained how the ratio is determined since as mentioned before the proportion of the chemicals used does not mean that the same ratio will be achieved in the vesicle. The electrode covered with a thioglucose layer served as the hydrophilic substrate. The preferred way (in my opinion) of vesicles fusion is used – simply prolonged contact of the electrode with the solution of vesicles. The Epzc of the electrode was established by the common immersion method, sometimes called Kolb method. What I miss in Fig. 4.3 is the same plot but for the layers containing the amyloid monomer and oligomer to see if there is any change of Epzc value (0.11V in the absence of amyloids). It may be the case based on what is shown in Fig. 4.9.

The capacitance changes and electrochemical impedance measurements were done for the bilayer films with and without monomers and oligomers of amyloids. The fBLMs were not ideal capacitors, however, the phase angles exceeded 80^{o} and a plateau was developed at low frequencies which indicated a highly capacitive system. The properties of the bilayers containing the amyloid monomers were similar, in contrary to the layers containing oligomers, for which a phase angle minimum was seen at the low frequency region. It reflects pores or defects formation. At positive potentials, the impedance of fBLMs was dominated by capacitance but for membranes with the amyloid oligomer the best fit was obtained assuming a water cushion underneath the lipid film and defects/pores in the layer. This impedance part, dependencies on the potential applied and discussion on the pore sizes at different potentials are really interesting and certainly present novelty in the studies of amyloid activity in the membranes. The PM-IRRAS experiments done at different potentials show that amyloid oligomers induce lipid disordering, reorientations of the acyl chains and a decrease in chains mobility. Based on more sophisticated 2D synchronous and asynchronous 2D-COS spectra, the Author came to conclusion that changes in the lipids preceded changes in the peptide itself.

Is there any way to inhibit the negative effects due to aggregation and pore formation by the amyloid oligomers? This issue was tackled in Chapter 5 by introducing a fluorine-based drug candidate, K162. The compound was found to decrease the amyloid oligomer toxicity *in vivo* but the mechanism of the process has not been explained. The Author postulates that in the presence of K162, the pathway of aggregation of monomers is changed and dimers are formed which next fibrillate instead of transformation to oligomers. The amyloid monomer was exposed to K162 either before or following its aggregation, and in addition to electrochemistry and AFM, molecular dynamics simulations were also performed to gain insight into possible K162 - amyloid β interactions. The impedance data revealed that K162 was present in the vicinity of the amyloid from the very beginning and when the vesicles were exposed to the K162-amyloid solution, the resulting bilayer at the electrode surface was much more impermeable and the phase angle did not show minima ascribed to pore formation. In addition, the constant phase element corresponding to the water spacer below the bilayer could be eliminated. The permeability of the lipid layer also did not increase with the addition of the K162 drug after aggregation to oligomer. The author interpreted these results as due to lower permeability of the membrane in the presence of the drug because of strong interaction between the drug and the amyloid. The AFM images confirmed that in the presence of the drug the globules and fibrils were formed instead of oligomers, therefore, the membrane structure remained unchanged. The MD binding energies reflect the role of hydrophobic residues in the aggregation of amyloids. In the presence of the K162 drug, the formation of oligomers is inhibited while fibrils formation is not prevented but these remain on the surface and are not toxic since they do not cause poration of the membrane.

The identification of the oligomers as the membrane destructing unit by the increased permeation mechanism as opposed to other forms of amyloids: monomers, dimers and fibrils was proved/confirmed by the three techniques used and is the main finding of this thesis. Revealing the role of hydrophobic interactions at a certain point of aggregation which explains the inhibiting role of K162 shown by the MD study is also an important contribution to the understanding of these complex processes.

It should be added that the Author did also a great job in terms of editing and preparing high-quality figures and images. Small mistakes similar to those:

Fig. 4.2: the proton at C3 is usually not shown,

Fig. 4.3: y axis is not charge density if in μFcm^{-2} ,

can be met from time to time in the text but there are only a few of them, and they do not disturb reading of the thesis.

In summary, an interesting and well written thesis is presented and huge and complex experimental work was accomplished by the graduate student. My minor critical comments and questions are mentioned just to clarify some points and warm up some discussion during the defense.

Based on the analysis of the doctoral dissertation, I conclude that the PhD thesis of Mr. Mrdenovic meets the conditions set out 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) and I am applying for the admission of Mr. Dusan Mrdenovic to further stages of the doctoral procedure. Moreover, the novelty of findings on aggregation pathways of amyloid β and oligomer interactions with membranes, obtained thanks to three methods that have not been used together in previous studies of amyloids, convinces me to apply also for awarding the doctoral degree to Mr. Dusan Mrdenovic with distinction.

W oparciu o przeprowadzoną analizę rozprawy doktorskiej stwierdzam, że spełnia ona warunki określone w art. 187 ustawy z dnia 20 lipca 2018r. Prawo o szkolnictwie wyższym i nauce (Dz.U. z 2018 r., poz. 1668 ze zm.) i wnioskuję o dopuszczenie Pana Dusana Mrdenovica do dalszych etapów przewodu doktorskiego.

Ponadto, nowość wyników dotyczących dróg agregacji amyloidu oraz mechanizmu oddziaływania z błonami komórkowymi uzyskana dzięki wykorzystaniu trzech metod, które nie były dotąd stosowane razem w badaniach amyloidów, skłania mnie także do zaproponowania wyróżnienia rozprawy doktorskiej Pana Dusana Mrdenovica.

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