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Referee report on the doctoral thesis by Dusan Mrdenovic

Author: Dusan Mrdenovic

Title: *Amyloid-β interaction with model cell membrane – insight into the mechanism of the Alzheimer's disease etiology and a novel therapeutic approach*

The presented doctoral dissertation of MSc Dusan Mrdenovic was realized under the supervision of Prof. Włodzimierz Kutner and auxiliary supervisor Dr Piotr Pięta in the Molecular Films Research group in collaboration with the group of Prof. Jacek Lipkowski at the University of Guelph located at the Institute of Physical Chemistry of the Polish Academy of Sciences (Warsaw, Poland), within the frame of the International Doctoral Studies in Chemistry.

The doctoral thesis mainly focused on the interaction of amyloid β (Aβ) with lipid membranes mimicking cell membranes in search of mechanisms involved in the permeation of membrane by specific amyloid forms and identifying their toxicity, followed by a fluorene-based active drug (K162) inhibition.

Neurodegenerative diseases affect millions of people worldwide. The risk of being affected by various types of neurodegenerative diseases increases dramatically with age. Moreover, they are incurable with a rapidly growing tendency of spreading as society ages. Alzheimer's disease is one of the most common examples of such diseases. It is associated with irreversible and progressive brain disorders that destroy human memory and thinking ability. In most people, the first symptoms appear around their mid-60s. Thus, it is critical to know and continuously improve our understanding of what causes this disease and how to link it with the development of new approaches for effective treatment and prevention. Various research carried out so far demonstrated that Aβ destroys the brain once it permeate the cell membrane. However, the knowledge of the exact mechanisms of the amyloid β with membranes is still not fully recognized. Such knowledge is essential for developing drugs that slow down this detrimental disease's progression.

In the last decade, new technologies have made it possible to research at the level of single cells or even molecules. Thanks to the obtained results, various detection systems are created

based on electrical, optical, mechanical, chemical, and biological phenomena. They can be applied to monitor disease-related alterations and deliver information on disease progression mechanisms, simultaneously being applicable to its detection, diagnosis, and treatment. The research carried out by MSc Dusan Mrdenovic is an essential part of the mainstream research on identifying the causes of Alzheimer's diseases and searching for possible means how to delay the disease progression. Already, the title of the doctoral dissertation itself fully reflects its content, at the same time indicating that the discussed topic of research is located at the border of medicine and chemistry. Such an interdisciplinary approach has a great potential to deliver results of great practical importance. It is worth emphasizing that the doctoral dissertation's research tasks were carried out based on international cooperation, which indicates a perfect preparation of the doctoral candidate to work in a team with various qualifications.

The doctoral dissertation is written in English. It is well-organized, well-documented, and well-written, making the thesis enjoyable to read. It clearly presents the research subject, objectives, and means to achieve them. Helpful lists of abbreviations and related papers authored and co-authored by Msc Dusan Mrdenovic are introduced at the beginning of the dissertation, followed by a clear summary (abstract).

The doctoral dissertation starts with the introductory part (Chapter 1), providing the background for understanding the importance of studying Alzheimer's diseases and the current state of the art. Although we know from the previous studies that amyloid β (Aβ) is a key player in developing Alzheimer's disease, there are still significant challenges to be overcome. The commonly well-established amyloid cascade hypothesis describing the Aβ aggregation pathway is not fully understood and described. This part of the Introduction is followed by state of the art and existing hypotheses on the interaction of Aβ with cell membrane, with the emphasis on the interaction of two Aβ forms, i.e., $A\beta_{40}$ and $A\beta_{42}$ peptides. The former is mostly abundant under normal physiological conditions, while the latter has been detected in patients who have Alzheimer's disease. $A\beta_{42}$ being detrimental to the cell membrane was the main subject of the study. The author of the presented doctoral thesis highlights its uncertainties and emerging questions still to be solved. Such a way of problem description requires from the candidate a lot of knowledge and insight into the research conducted so far. Indeed, the attached bibliographic list (374 positions), along with references cited in the already published scientific papers, testifies to the extensive study of the subject and the Ph.D. student's commitment to the doctoral dissertation subject. This part of the thesis ends with the clearly defined thesis objectives. Chapter 1 is followed by Chapter 2, explaining the methods and analytical approaches used to realize the thesis objectives. The author applied a wide range of well-chosen techniques, such as atomic force microscopy (AFM), electrochemical impedance spectroscopy, infrared (IR) spectroscopy, or differential capacitance measurements, accompanied by methods used to prepare lipid membranes (e.g., supported lipid bilayer preparation). Electrochemical methods provide information about membrane integrity that supplement the AFM results, while infrared (IR) spectroscopy is an

excellent technique used to study the sample structure and chemical properties. The description is clear and covers the basics of the techniques applied at the level enabling us to understand the obtained results. The use of these techniques in the study of amyloid formation and mechanisms of its interaction with lipid membrane without or with the presence of inhibitor is not trivial and required more effective mastering not only the state of knowledge but also the methods of sample preparation in such a way that their structure and biological activity were not damaged.

The next three chapters present the results obtained during the thesis realization. Two first were prepared on the basis of the already published papers in international journals, i.e., in *Langmuir* and *Nanoscale Advances*. The third includes the unpublished yet results but from the submitted paper. Studies mainly involved on the detrimental $A\beta_{42}$. Chapter 3 is devoted to the mechanisms responsible for the amyloid β oligomers (AβO) toxicity. To unravel it, MSc Dusan Mrdenovic introduced AβOs of controlled size and concentration into a model cell membrane with lipid composition similar to membranes occurring in the brain. By studying surface topography and nanomechanical properties using atomic force microscopy (AFM), it was possible to distinguish the mechanisms involving of small-size (SS) and large-size (LS) AβO. The high-resolution AFM images show that LS AβOs fibrillate through both primary and secondary nucleation mechanism on the lipid surface (brain total lipid extract, BTLE) without affecting the membrane integrity. Opposite mechanisms were observed for SS AβOs that destroy the membrane first by forming pores and then lipid extraction. Chapter 4 complements the AFM-related studies with the information on the chemical and electrical properties of model membranes, in which AβOs induced defects. Their presence results in a significant change in the membrane electric properties resulting from its poration caused by AβOs. Both AβMs and AβOs cause conformational and orientational changes of lipid molecules in parallel. Their interaction with the hydrophobic core increases the tilt angle of lipid acyl chains, increases the number of gauche conformers in the lipid acyl chains, decreases lipid acyl chains' mobility, and changes the lipid molecule packing. The obtained results showed that the interaction of AβOs with the membrane is stronger than AβMs. Chapter 5 demonstrates the effect of a specific Aβ inhibitor, i.e., K162 – a fluorene-based compound. Although it has been reported that this inhibitor decreases AβOs toxicity *in vivo*, the mechanisms leading to these effects were not described. The study included in the presented doctoral thesis reveals that K162 prevents AβOs-induced bilayer lipid membrane permeation by altering the Aβ aggregation pathway. The results show that in the presence of the K162-modified Aβ aggregation, AβMs dimerize and fibrillate. This way, the formation of membrane-permeating AβOs is impaired, and more importantly, K162 preserves the neurologically beneficial AβMs. The last part of the thesis is a summary chapter that includes a list of open questions still to be resolved. An extensive list of bibliographic positions follows this part.

Making summary, during the reading, the readers obtain a nicely written (also from an editorial point of view), in-deep and comprehensive story on how amyloid β destroys cell

membrane contributing to the further progression of Alzheimer's disease, which end up with a positive ending showing that the use of Aβ inhibitors prevents the detrimental effect Aβs. Thanks to systematic and careful studies, novel information showing the mechanisms of the Aβ-induced changes in the model membranes is shown. This will help to understand better the mechanism of interaction of Aβ with bilayer lipid membranes and, consequently, pathology of Alzheimer's disease. Moreover, the presented doctoral thesis is the examples supporting the thesis that the development of new biophysical methodologies, which can bridge together the investigation on morphological and ultrastructural properties of amyloid at various scales, is needed for a fruitful avenue to address various challenges in amyloid-related diseases. Such a molecular-level understanding of the key pathogenic processes is crucial to provide the answers to still open questions that will help establish rational approaches to the prevention and design of pharmacological approaches to combat such diseases.

While reading the paper, the only a few comments/questions arose:

- Chapter 2 describes the techniques in a general way. At the end of each section paragraph, I would expect a short description of the instruments used in the preparation of the doctoral thesis. I would also include short information on NMR, as this technique complements the research included in Chapter 3.
- From the AFM point of view, it would be good to include the equation used to deconvolute molecules' size.
- As is nowadays discussed widely, the assessment of nanomechanical properties of various materials using the Hertz model is a bit questionable due to mainly geometry of the probing tip and sample materials. I wonder what the prerequisites for applying the Hertz model in the presented studies are. Can the end of the cantilever be considered as a sphere?
- In the case of thin samples, the effect of the underlying stiff substrate can strongly affect the obtained results. Did the authors apply the finite thickness correction?
- Nanomechanical characterization of biological systems by AFM also encompasses microrheological measurements, in which a transition between elastic and viscoelastic behavior can be obtained. Is this also applicable to model cell membranes?
- I feel a bit unsatisfied with the part devoted to Aβ inhibitors. The author chosen the K162, a fluorene-based compound re-calling the paper from 2010. Is this compound in clinical use right now? Is it the only compound that preserves the AβMs consumption? Do the novel, more effective inhibitors exist?

The noticed inaccuracies do not diminish the substantive value of the doctoral dissertation, which is of the high professional level. In the summary of my review, I state that the doctoral thesis of Dusan Mrdenovic is an innovative and original approach to research on the role of Aβ in Alzheimer's disease progression. The obtained results are valuable not only for understanding the mechanisms of the Aβ interaction with lipid membrane, but they also help

put a different perspective on drug design research. The presented results are original, have the features of scientific novelty, and, most importantly, open up further research opportunities.
Scientific achievements of MSc Dusdan Mrdenovic resulted in 5 published/submitted scientific papers that are strongly linked with the subject of the Ph.D. thesis. In these papers, the candidate is the first author. Apart from this, the candidate co-authored three other papers and presented results in three conferences.

Taking into account all elements of the review, I conclude that the dissertation meets all requirements set for doctoral dissertations by the Act on Academic Degrees and Title (*art.187 ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce, Dz.U. z 2018r., poz. 1668 ze zm.*) and by Academic Standards, and I recommend MSc Dusan Mrdenovioc to be admitted to further steps in the procedure of the doctoral dissertation defense. Simultaneously, the immense topicality and great practical importance of the obtained results increase the value of the work. Considering this, I am asking the Scientific Council of the Institute of Physical Chemistry to award (summa cum laude) the doctoral dissertation by Dusan Mrdenovic (the justification is included in a separate document).

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The justification for the doctoral dissertation of MSc Dusan Mrdenovic award (summa cum laude)

Amyloid β is linked to incurable Alzheimer's disease affecting older people, simultaneously causing a global health problem. Therefore, knowing the exact mechanisms explaining how this protein effect membranes present in the human brain is of uttermost importance. Various discoveries have contributed to our partial understanding of Aβ oligomerization, toxicity, and interaction with cell membrane; however, our knowledge is not complete impairing the development in designing new drugs slowing down the detrimental effects of Alzheimer's disease. In lime of this, the importance of the studies included in the doctoral dissertation presented lies in:

- Unraveling the mechanism of Aβ aggregate permeation through cell membranes in physiologically-relevant model.
- Identification of the Aβ aggregate type actively participating in permeation process
- Deciphering morphological and structural characteristics of the toxic form of Aβ aggregate that are associated with the structural disorders affecting the nanomechanical, electrical, and chemical properties of model membrane damaged by Aβ aggregates
- Demonstration that amyloid β oligomers can be inhibited by K126 compound with simultaneous preservation of its monomeric/dimeric forms.

Identification of the Aβ-induced lipid membrane permeation mechanism is necessary to understand Alzheimer's pathophysiology and develop an effective therapeutic strategy against it.

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