

**Review of the doctoral dissertation of Ms. Natalia Szulc, M.Sc. entitled
„Intrinsic and extrinsic determinants of the aggregation process of amyloid proteins”.**

**Discipline: biomedical engineering at Wrocław University of Science and Technology;
chemistry at l'Université de Lorraine**

General remarks on the issues raised in the Doctoral Dissertation

Amyloids are highly ordered cross- β sheet protein aggregates associated with many diseases including human prion, Alzheimer's and Parkinson's diseases, but also with biological functions such as hormone storage. Their three-dimensional structure is unique among protein folds because it folds only upon intermolecular contacts (for folding to occur, only short sequences of amino acid residues are required), as well as the structure repeats itself at the atomic level (i.e., every 4.7 Å). It should be noted that amyloids are sensitive to various experimental conditions in which their aggregation process is studied.

The evaluated dissertation presents original research results in the area of biomedical engineering and chemistry. The thesis is focused on selected internal and external factors, which may affect the aggregation process of amyloidogenic peptides. Furthermore, it raises potential problems of the ambiguity of results from experimental techniques in amyloid studies. The dissertation submitted for review is written in English. The thesis was prepared at the Wrocław University of Science and Technology (Department of Biomedical Engineering) and Université de Lorraine, École doctorale Chimie - Mécanique – Matériaux – Physique under the supervision of Prof. Małgorzata Kotulska, PhD, Eng and Dr. Maunir Tarek. Thesis supervisors are competent and well-selected scientists, who have made groundbreaking contributions to the field of computational chemistry, biophysics as well as molecular modeling. Scientific achievements of Ms. Natalia Szulc, M.Sc. are significant; she is already an author of 8 publications and she took part in 2 externally funded projects (*InterDok – Interdisciplinary Doctoral Studies Projects at Wrocław University of Science and Technology, co-financed by the European Union under the European Social Fund*

no. POWR.03.02.00-00-I003/16; Bourses du Gouvernement Français – Doctorat cotutelle fellowship, Campus France).

The PhD Candidate endeavored to study the effectiveness of the protocols and the dependence of the results on the experimental conditions. The research was conducted with a focus on the following: solvent and influence of ions: presence, type and concentration; sequence determinants (mutations); the lipid membrane environment; interaction with another peptide. The studies were carried out considering: various variants of hexapeptides from well-known proteins, which were the base data for very many bioinformatics predictors; fragments of the CsgA (consisting of R1-R5 imperfect fragments) functional amyloid protein derived from *E. coli* and *S. enterica* bacteria; mutant peptides of the R4 fragments of CsgA protein from *E. coli* and *S. enterica* bacteria; amyloid- β 42 (A β 42) and human islet amyloid polypeptide (hIAPP) also called amylin.

Such studies are at the forefront of the development of modern and creative therapeutic tools. Recently some results of this thesis were published in a remarkable paper in *Nucleic Acids Research* (M. Burdukiewicz, D. Rafacz, A. Barbach, K. Hubicka, L. Bąkała, A. Lasota, J. Stecko, N. Szymanska, J. W. Wojciechowski, D. Kozakiewicz, N. Szulc, J. Chilimoniuk, I. Jęśkowiak, M. Gąsior, Głogowska, M. Kotulska, (2023) *AmyloGraph: A comprehensive database of amyloid-amyloid interactions*, *Nucleic Acids Research*, 51 (D1), D352–D357).

The assessment of this thesis can only be positive. The presented research is carefully designed and very thoughtful. Notably, Ms. Natalia Szulc, M.Sc. published two first-author papers, which included some other results of her thesis in respectable journals: *Scientific Reports* and *International Journal of Molecular Sciences*.

The experiments are very well planned and executed. The text itself, including the writing and editorial side is almost flawless.

Substantive and editorial evaluation of the Doctoral Dissertation

The dissertation title precisely defines the scope of the work. The dissertation comprises 159 pages and does not follow the classic structure of a PhD thesis in science. It begins with abstracts (in English, French and Polish) and an introduction to the subject, which directly leads to a list of four research hypotheses. The introduction gives the reader a very good overview of the field, with just a sufficient amount of detail to help understand the rationale of the doctoral work.

The introduction consists in a literature review, has a logical partition and includes the fundamentals of protein aggregation process and a description of intrinsic and extrinsic factors affecting amyloids' aggregation as well as the main experimental methods employed in amyloid studies. The literature for this part is carefully referenced (157 references) and a comprehensive bibliography contains all the relevant papers for the discussed field. It is worth mentioning here that most of the references are from the last decade, showing the topical issues. This part is well written and provides a very good background for understanding the rest of the thesis. The remaining chapters of the doctoral thesis present the research hypotheses, materials and methods, results, summary as well as future perspectives.

The work carried out within this thesis aims at addressing the following research hypotheses: (i) minor deviations in experimental conditions strongly influence the validity of a protein classification as amyloid; (ii) aggregation propensity of closely related homologous functional amyloids may be different; (iii) the presence of a lipid membrane affects aggregation of the native A β 42 peptide; (iv) the presence of another peptide/protein impacts the general fibrilization process. These hypotheses may seem rather obvious, nonetheless they should be verified by a scrupulous study which is the subject of the thesis. The experimental chapters are clearly written and their merits testify to the excellent preparation of the student for the research work.

The methodology adopted in the dissertation is appropriate. Circular dichroism, FT-Raman, vibrational spectroscopy, atomic force as well as transmission electron microscopy, off-null detection scanning Kelvin probe and computational methods have been utilized as the main tools for the detailed study. The 'Materials and Methods' section is well prepared. I have no doubts that the presentation of procedures/methods presented contains sufficient information to allow for the complete reproduction of experiments by other researchers.

The presented results seem to be reliable and well documented, although, as always in cutting edge research, the interpretation as well as discussion can be extended and more in-depth. The results of the research demonstrate that it is important to collect coherent data from experiments, which could be disrupted by non-identical conditions. Furthermore, different experimental methods may deliver somehow different results regarding the same peptides. This effect is more significant in the case of some sequences. Ms. Natalia Szulc, M.Sc. and coworkers found that short hexapeptide sequences exhibit distinct aggregation propensities in response to external factors, e.g., the solvent used, compared to longer sequences (up to 23 amino acids).

The flexibility of six-amino acid sequences allows them to adopt specific conformations. The phenomenon of symmetry-breaking transitions plays a crucial role in this process. What is more, Natalia Szulc's studies showed that the choice of solvent influences the aggregation process. The usage of deuterium oxide might alter the classification, as observed for the R2 fragment of *S. enterica*. The Candidate showed that R4 fragment from *S. enterica* (SR4) has a larger tendency to aggregate compared to the R4 fragment from *E. coli* (ER4). Theoretical sequence analysis revealed that SR4 has higher hydrophobicity and electric charge values than ER4. Those factors are known to increase protein aggregation. In the last phase of the study, the stability of A β 42 in a lipid membrane was examined through molecular dynamics simulations and atomic force microscopy.

In general, the study sheds light on the potential challenges arising from the ambiguity of experimental outcomes in the context of amyloid investigations. The research has shown that even small changes in experimental conditions can alter the properties of amyloid peptides and proteins, which can be the cause of obtaining incorrect models and predictions of bioinformatics tools, based on incompatible learning data. However, as shown in the study, minor deviations are not detrimental to these tools.

In the final section, the general conclusions of the work are summarized. The results of the study indicate the importance of collecting consistent data from experiments conducted under various conditions. Ms. Natalia Szulc, M.Sc. suggests that bioinformatics predictors are quite resilient to data incompatibilities, but their performance may be disrupted if the influence of experimental conditions and insignificant differences in homologous sequences are not taken into account. It is essential to thoroughly acknowledge the non-identical nature of experimental conditions. The results of the research and their discussion are well documented by many elaborated drawings, images, graphs and tables. The thesis is prepared in good editing standards. All the figures are carefully prepared and clearly presented. The language is comprehensive and coherent while errors and inaccuracies are relatively rare. The presented work clearly shows the Candidate's ability for creative scientific work.

Editorial remarks

I have a few minor editorial remarks:

It is not obvious how sterility of the samples was maintained during prolonged (up to one year) incubation.

Page 28: why "to make this solution", unclear;

Page 28: “kinetics and morphology of formed fibrils”, kinetics is irrelevant for formed fibrils;

Page 28: “lysozymes” should be replaced by “lysozyme”;

Page 29: “various biological machinery”, the phrase is somewhat unclear;

Page 31: “leading for instance to ionic homeostasis”, apparently the author meant disturbance of ionic homeostasis;

Page 47: It is unclear how was it possible to obtain pH =7 by diluting 0.1 M NaOH with PBS, pH 7.2;

Page 89: “even tough”, I guess the author meant “even though”.

Perhaps most of these minor uncertainties are due to the translation of the thesis from the original language into English.

The above-mentioned inaccuracies or minor errors do not affect the scientific value of the dissertation. The objectives of the work have been successfully achieved.

Final evaluation statement

In summary, the dissertation presented to me for evaluation describes research at a high methodical and scientific level. I believe that the dissertation meets the conditions set forth in Article 187 of the Act “Law on Education and Science” of July 20, 2018 so with full conviction I propose to admit Ms. Natalia Szulc, M.Sc. to further stages of the doctoral procedure. At the same time, taking into account the high quality of the dissertation, the high level of the presented research, a very important field of research and publication success, I propose to award the dissertation with an appropriate distinction.

Rzeszow, June 8, 2023

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