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Review of Doctoral Thesis MSc Manuel David Peris Díaz "Biophysical characterization of metallothionein Zn(II)-loaded states and their role on cellular zinc buffering".

Presented work was performed by MSc. Manuel David Peris Díaz under the supervision of Prof. Artur Krężel from the Department of Chemical Biology, Faculty of Biotechnology, University of Wrocław. Considering the high challenge of structural study of metallothioneins, the candidate undertook the ambitious task of designing an analytical methodology based on chemical labelling and mass spectrometry, developed data analysis software for MS data analysis and combined this data with chemometric and molecular dynamics analysis. All this is to discover the molecular bases for the origin of the Zn(II) binding affinities to metallothioneins.

The doctoral dissertation is written in English based on published results in five high-rank journals. In all papers, MSc. Manuel David Peris Díaz is the first author; in one of them, he is also a corresponding author. Overall, the presented thesis is innovative and supported by five first-author peer-reviewed publications with a total IF 44.534, 680 MNiSW points, and 42 citations. It is worth emphasizing that the results were published in 2020 and 2021. The review was published in Trends in Analytical Chemistry, where the impact factor is 12.296 (140 points on the MNiSW scale), and the work with the

Journal of the American Chemical Society with IF 15.419 and 200 points on the MNiSW scale. This work was also selected for the cover of the JACS issue.

The thesis is organized in sections covering the abstract, list of abbreviations, introduction, research aims, five chapters dedicated to each publication included in the thesis cycle, summary and outlook, conclusions and an appendix with candidate curriculum vitae. Extensive literature citations support all. The introduction (20 pages) describes zinc metalloproteom, mammalian metallothionein and their structural studies. The last four paragraphs of this section are devoted to mass spectrometry in the context of metallothionein research, computer simulations and chemometric-assisted electrochemical research in these proteins. This part of the work perfectly introduces the reader to the topic of the work. The aims are presented clearly and concretely in Chapter 2 (Research aims).

The following section is the Results and Discussion and consists of five chapters. Each of them is devoted to one publication that makes up the series. Very original was to add a fragment described by MSc. Peris-Díaz as "Bibliometric data and brief history", these few sentences about the origin of the concept, its evolution, and also self-criticism of each of the publications gave the work a light personal touch. A summary, usually one and a half to two pages long, summarises the material from the publication. Chapter 8 contains conclusions regarding the entire dissertation, and the work ends with an appendix in the form of the author's CV with a list of publications and acknowledgements. The whole manuscript ends with a list of references used in the thesis (244 literature items). The work has been carefully prepared for editing, and I have no comments on this element. There are a few typos that do not need to mention.

After reading the thesis, I can conclude that the objectives set by the candidate presented in Chapter 2, such as understanding the Zn (II) ion binding mechanism by metallothioneins, elucidation of the spatial organization of zinc in the Zn₁₋₇MT species and

determination of the molecular basis for the differences in Zn (II) binding affinities to MT were achieved

I believe MSc Manuel David Peris Díaz has extensive experience obtaining research funds, planning and conducting experiments, and publishing results and is fully ready for further independent research work.

The candidate can also boast many awards and scholarships, such as the Max Born Award from the mayor of Wrocław (2019), the prize for young researchers in Poland from the Minister of Education and Science (2021-2024), and the FNP START scholarship (2022). He also obtained research funding from a Preludium grant from the National Science Center. He carried out his work not only in Wrocław but also at the University of Brno, under the supervision of Professor Vojtech Adam, the University of Manchester, under the supervision of Professor Perdita Barran, and the University of Utrecht under the supervision of Professor Albert Heck. These scientists have contributed to the development of mass spectrometry and proteomics, so one can only envy such internships.

A few questions for the candidate:

- Can you estimate the yield and purity of the final protein (MT2 and MT3) product for experiments?
- Have there been attempts to obtain synthetic MT proteins?
- Was a buffer other than ammonium acetate checked for native spectra?
- In the publication from Analytical chemistry in Table S2. Gas phase ions annotated for Zn₄MT₂ labelled with IAM. Product ions were annotated as [M+H]⁵⁺ adducts. The calculated and experimental m/z values refer to average mass. Also, in table S1 from JACS, there are differences in mass obtained after deconvolution of ESI spectra for complexes apoMT₂ with Zn(II) reaching 2.9 Da. The MS spectra are presented in Fig. 4A. Why such a difference in the theoretical and experimental mass for

$Zn_4IAM_6MT_2$ (calculated 1330.77 m/z, experimental 1327.31 m/z)? What could be the reason?

In conclusion, I would like to state that the Doctoral thesis presented by MSc Manuel David Peris Díaz fulfils all requirements needed to obtain the Doctoral degree in Biological Sciences following the relevant legal regulations and customary standards. Therefore, I recommend that the Scientific Council of the Biological Sciences of the University of Wroclaw admit MSc Manuel David Peris Díaz to further steps towards the degree of Doctor of Biology (PhD). Also, considering the high quality of the obtained results and their publication in prestigious journals, I nominate this Doctoral thesis for the appropriate award.

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