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Review of the doctoral dissertation by Sakshi Sareen, MSc, entitled „Biophysical Symptoms of Cellular Stress"

Stress response is one of the key capabilities of living systems that enables their survival and evolution. Understanding the mechanisms governing stress response towards different stimuli can not only broaden our comprehension of life itself but also guide the development of novel diagnostic and therapeutic approaches. Investigating the stress response at the biophysical level is the core of the dissertation of Sakshi Sareen, entitled „Biophysical Symptoms of Cellular Stress", carried out under the supervision of Prof. Robert Holyst and Dr. Karina Kwapiszewska at the Institute of Physical Chemistry, Polish Academy of Sciences, Department of Condensed Soft Matter.

The dissertation investigates the effects of cellular stress, particularly starvation and mitochondrial dysfunction, on the biophysical properties of cancer cells, utilizing advanced biophysical techniques such as Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Lifetime Imaging Microscopy (FLIM). The work focuses on quantitative analysis of intracellular diffusion, viscosity, and autofluorescence, and employs CRISPR-Cas9-based gene knockouts to investigate stress responses at the molecular level.

The doctoral dissertation is 83 pages long and follows a structure typical for experimental scientific works, including a brief introduction, a results section subdivided into key experimental chapters, each followed by a short summary, and a final summary with conclusions. Two appendixes include a list of abbreviations (app. A) and detailed experimental procedures (app. B). The work begins with clearly defined research objectives and is preceded by both an English and Polish abstract.

The dissertation is well-organized and carefully edited. It includes 37 informative figures and about a dozen tables, which are of high quality and significantly enhance the clarity and scholarly presentation of the data. The thesis cites 158 bibliographic references, including all literature relevant to the fields of cellular biophysics, stress response, cancer research, and gene editing.

The subject of my assessment of the thesis includes the following elements of the dissertation: a) the Authors' general knowledge in the field of science, b) the ability to conduct scientific research, c) the originality of the scientific problem solved.

In the literature section of the dissertation, the Author discusses the biological relevance of cellular stress and its impact on cell physiology, with particular emphasis on biophysical responses such as macromolecular crowding, intracellular diffusion, viscosity, and autofluorescence. Special focus is given to two major stress conditions: cellular starvation and mitochondrial stress, highlighting their effects in both healthy and cancerous cells. The author reviews the mechanisms of stress response across various organisms, from bacteria and yeast to mammalian cells, and highlights the adaptive strategies cells employ to maintain homeostasis under unfavorable conditions.

Considerable attention is also devoted to the biophysical properties of the intracellular environment, including diffusion dynamics and nanoviscosity, as well as how these are measured using techniques such as Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Lifetime Imaging Microscopy (FLIM). The author further discusses the role of autofluorescent molecules (e.g., NADH and FAD) as metabolic indicators and describes the limitations and advantages of various ATP detection methods, including newly developed sensors. The introduction concludes with an overview of CRISPR/Cas9 gene editing, highlighting its utility in generating knockout models for studying intracellular transport and stress response. The literature review presented is brief, yet helpful and well-integrated with the dissertation's main topic. It provides a solid foundation for understanding the research rationale and supports the methodological choices made in the Author's experimental work.

The Results section is structured into multiple experimental chapters, each investigating a distinct but interrelated aspect of biophysical changes in cancer cells under stress. In the first part of the research, the Author investigates the impact of cellular starvation on intracellular diffusion, using GFP as a molecular probe. These studies, based on FCS measurements under nutrient-deprived conditions, reveal a progressive increase in diffusion hindrance, particularly in the cytoplasm of HeLa cells. This is complemented by a parallel study of ribosomal subunits under starvation, demonstrating how molecular size affects intracellular mobility under stress conditions. The Author uses theoretical models and quantitative parameters (e.g., nanoviscosity and diffusion hindrance factors) to rationalize the findings.

In the next part of the dissertation, the Author examines the biophysical consequences of knocking out the CLCNKA gene, encoding a voltage-gated ion channel, using CRISPR-Cas9. After preparing the cells, the Author compared the GFP diffusion properties of wild-type and knockout cells under identical starvation conditions. Since the knock-out cells did not show a similar response to starvation as the wild-type cells, this set of experiments demonstrated the link between the function of the chloride channel and cellular diffusion, as well as volume homeostasis.

The next chapters are devoted to the evaluation of ATP depletion and mitochondrial damage-induced autofluorescence. The Author explores the possibility of quantifying intracellular ATP levels using a recently developed small-molecule ATP probe, bisantrene. Unfortunately, the probe proved unsuitable for measurements in such a complex environment due to issues with solubility and cytotoxicity. The analysis of staurosporine-induced mitochondrial stress completes the thesis: the Author tracks changes in autofluorescence across multiple cancer cell lines using FLIM, demonstrating enhanced autofluorescent signals consistent with apoptotic processes. The work culminates in a meticulous fluorescence lifetime analysis that reveals a shift toward longer autofluorescence lifetimes in response to a high level of mitochondrial damage.

Taken together, the research presented in the thesis tackles a set of essential problems linked to cellular stress response. It is characterized by methodological rigor, a diversity of experimental approaches, and the careful execution of experimental tasks. The results are presented in a clear and easy-to-follow manner. Their interpretation is very careful and not overreaching. However, in my opinion, the interpretation of the results sometimes appears too narrow, which raises slight dissatisfaction. A broader interpretation, highlighting the significance of the results in the biological context, would be even more informative. The work has been carried out at the intersection of biophysics, cell biology, and genetic engineering, employing a variety of experimental techniques, including FCS, FLIM, and HPLC, in conjunction with theoretical calculations. One of the most notable achievements of this work, in my opinion, is the demonstration that biophysical parameters such as intracellular diffusion and viscosity can serve as sensitive, quantifiable markers of cellular stress.

Several issues raised in the dissertation have aroused my interest, and, driven mostly by my curiosity, I would like to ask the author to clarify them during the public defense:

1) Page 25 – measurements in the presence of YO-PRO1. The author mentions that the dye is a DNA intercalator, which also interacts with double-stranded RNA (dsRNA). Please, explain how this property enables ribosome tracking? How is the signal from ribosomes distinguished from cellular nucleic acids? Why are ribosomes exclusively labelled and no other proteins?

2) Regarding the sentence "In nutrient-deprived conditions, cytoplasm might undergo a change in pore size, decreasing from 100 nm to 30 nm." Please clarify whether this is a literature finding or a suggestion based on the experimental results. If the second, how did the author arrive at this conclusion?

3) The author writes: "The diffusion coefficient obtained (~18 μm2 /s on day 1) after fitting the data with 1 component model seems too big for a molecule of 15 nm in size. Along with photobleaching, this could indicate the immobilisation of LSU in starved cells, as predicted. YO-PRO-1 also binds to tRNA (2 nm), but because it is a small molecule (smaller than GFP – 2.3 nm), its expected diffusion coefficient should be higher." Did you consider free ribosomal subunits only in your model? How does it correspond to the fact that ribosomal subunits form complexes in the cells (with each other, proteins and mRNA; e.g. initiation complexes, monosomes, polysomes)? Do these structures not interfere with the measurements? Taking into account their existence how do you justify adopting only a 1-component diffusion model? Also, what is the potential cellular mechanism of "immobilization" that you mentioned while discussing the results?

4) "It was observed that the changes in diffusion hindrance were more pronounced for 40S ribosomal subunits (3.75 nm) than those of GFP (2.3 nm) (Fig 9). This suggests how a small change in probe size affects the molecular mobility inside cell cytoplasm under extreme stress conditions such as prolonged starvation (Fig 9)." Is this possible that the difference occurs due to some biological processes involving ribosomal proteins under stress conditions? (If so, please speculate).

5) Experiments with 769-P cells. Please explain in more detail why this particular cell line was used as a model (HeLa cells used in the earlier experiments are also cancer cells).

6) The conclusion that starvation affects only cytoplasmic, but not nuclear diffusion, is very interesting. Has this ever been observed before (i.e. reported in the literature)? Can the author speculate what the putative biological meaning of this phenomenon is?

7) What could be the reason (at the molecular level) that the HeLa cells do not respond to stress in the same way after knocking out the CLCNKA gene?

8) Figure 21 shows putative interaction of bisantrene and ATP molecule. Is the depiction accurate? Please explain what π-π stacking is and which part of the ATP molecule stacks against the π-system of the sensor?

9) The literature paper on bisantrene reports it is a very selective turn-on probe for ATP in water (https://pubs.rsc.org/en/content/articlelanding/2019/cc/c8cc09857c). In contrast, the data shown in Figure 24 suggests the opposite (turn-off-like behavior). How do you explain this? What is the main putative mechanism for cytotoxicity of bisantrene? Have you tried lowering its concentration for cell culture measurements?

10) Figure 33. In the trace of FAD standard there are two larger peaks (~7.5 and 10.5 min) in addition to the peak labeled as FAD. Please explain what is their source of origin?

11) A significant portion of cellular FAD is tightly bound to proteins. Is it possible that the increased autofluorescence in some cases is due to the aggregation of these FAD-bound proteins (and not free FAD molecules)?

Importantly, a significant portion of the research presented in the thesis has already been published (two published papers, two under preparation). Particularly noteworthy is the work published in the journal Nanoscale, in which the Author of the thesis is the first co-author, and which encompasses the majority of the studies related to FCS measurements of diffusion under stress. I also find it impressive that the Author took the effort to publish a comment in *ChemComm* on the unsuitability of bisantrene for more advanced applications. Scientists often encounter that published results either do not reproduce well or are not as universal as depicted. Reporting such cases is always a great service to the scientific community, as it saves time and money for other researchers.

Overall, the dissertation demonstrates a high level of experimental skill, analytical depth, and scientific maturity, making a valuable contribution to the understanding of biophysical stress responses in cancer cells. I am confident that the experimental results described in the thesis advance the field of cellular biophysics and expand the available repertoire of qualitative and quantitative tools for monitoring cellular stress responses. The design of the study is very well thought out, the execution is carried out with the aid of advanced state-of-the-art experimental approaches, and the results open new avenues for the quantitative assessment of stress-related biophysical parameters. The findings of this thesis, along with the developed experimental approaches, will be beneficial to other scientists worldwide and may contribute to the development of novel diagnostic tools. In summary, I am fully convinced that the doctoral dissertation submitted for my assessment meets all the conditions specified in the Act of July 20, 2018, on higher education and science (Journal of Laws of 2018, item 1668, as amended), the Act of July 3, 2018, Introductory Provisions to the Act on Higher Education and Science (Journal of Laws of 2018, item 1669, as amended), and I appeal to the Scientific Council of the Institute of Physical Chemistry of the Polish Academy of Sciences to admit Sakshi Sareen, M.Sc., to the next stages of the doctoral procedure

Sincerely,

Joanna Kowalska

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