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**Review of the doctoral dissertation of Izabela Stupka "Engineering of protein cages for biomedical applications".**

This thesis describes the development and characterization of protein cages with large potential for biomedical applications. Such studies are at the forefront of the development of modern and creative therapeutic tools. Cages can be used for example for targeted and controlled drug delivery. They are already used for vaccine production. The supervisor of this thesis has made groundbreaking contributions to the field by discovering a new type of protein cage assembled through interaction of protein rings with gold ions (so-called TRAP-cages). The key feature of this cage is their reversible assembly. These results were published in a remarkable paper in Nature in 2019. This was an exciting development in the field and the presented thesis builds up on this work.

The assessment of this thesis can only be positive. Presented results greatly expand the possible designs of the TRAP-cages with particular emphasis on controlled disassembly. The experiments are very well planned and executed. The text itself, including the writing and editorial side is flawless. Last but not least, a part of the results in this PhD project concerning the maleimide cross-linker cages was published in 2022 in Science Advances which is a journal with growing recognition for publishing high-quality work. In this publication Izabela Stupka is the first author.

The introduction presents various protein cage designs: from natural cages to designing artificial ones. Cage engineering approaches are also described. Finally, methods of loading the cages with other molecules (encapsulation) are presented. The figures included in the introduction greatly help in following the text. The introduction gives the reader a very good overview the field, with just sufficient amount of detail to help understand the rationale of the doctoral work.

The initial goal of the project was to design cages which can be disassembled in a controlled fashion. The idea was to use maleimide cross-linkers to form the cage. Cleavage of these cross-linkers by various factors could lead to cage disassembly. The initial step in this project was to design TRAP-cage variants with cysteine residues at the interface between protein rings that constitute the cage. These cysteines could react with homo-bifunctional maleimide cross-linkers. One of them comprised a disulfide bond which could be reduced (cleaved) at will. Izabela successfully showed that at least

for some bifunctional maleimide linkers TRAP cages can be obtained. Another approach was to use bromine compounds which are known to react with sulfhydryl groups. Here, the formation of the cages required their initial assembly in the presence of Au ions, which was a novel and creative experimental approach. Two cages formed in the presence of maleimide cross-linkers were analyzed structurally by cryo-EM confirming their structure and even visualizing the linkers themselves.

The best maleimide-based cages were thoroughly characterized. For example for the DTME and BMH cross-linkers, it was shown that they can withstand temperatures up to 75°C and very harsh chemical conditions with 7 M urea or 7% SDS, showing their remarkable stability. Importantly, the cage with disulfide-containing DTME cross linker readily disassembles in the presence of reducing agents.

The next step was to devise methods to package the cages with model proteins. Two fluorescent proteins were chosen as model cargoes. They were fused to the protein building blocks of the cage and co-expressed in with unmodified building blocks controlling the amount of each protein form. The structural integrity of the cages was confirmed and simultaneous packaging of both proteins to the cages was verified by observing FRET between them. FRET measurement could also be used to follow the disassembly of the cage in the presence of the reducing agent DTT. These results showed that the Au-stabilized cages and DTME-stabilized ones have slightly different disassembly kinetics.

The use of reducing agent for cage opening is difficult to apply in actual medical applications. Physical factors such a light or magnetic field would be much more useful. Their advantage is also that they can be applied in a spatially and temporally controlled fashion. Izabela focused on developing light-disassembling (photo-cleavage) cages. For that she revisited the bromine-containing cross-linker with nitrobenzene scaffold, in particular 1,2-BBN. Cysteine cross-links formed by this compound should be cleaved upon UV irradiation. Appropriate cages were formed and, indeed, they were disassembled by UV light. However, this reaction was reversible. Another clever trick used in this work was the addition of DTT as radical scavenger which prevented the reformation of the crosslink.

UV is not an ideal factor for triggering the cage disassembly in medical applications. It is harmful to cells and has very small penetrance. Therefore Izabela wanted to develop other bromine-contains cross-linkers which can be cleaved by longer wavelength light (here the ideal stimulus would infrared light which has high penetrance and is largely innocuous). To this end Izabela used other bromine-containing cross-linkers which should be photolabile at shorter wavelengths (blue light) but none of these cages could be disassembled by blue light.

During the defense I would like to Izabela to discuss possible approaches (even very creative and unorthodox) for creating cages which would disassemble upon irradiation with long-wavelength light. What other physical factors could be used there and how to implement their effect in these systems?

The next part of the thesis describes an alternative method of cage loading through SpyCatcher-SpyTag combination. This protein-peptide pair react with each other forming a covalent link. SpyCatcher was attached to the cage components and cages were made by co-expression with unmodified cage building blocks as described above. The Spy-tag was attached to interleukin 2 (IL-2) and its variants. IL-2 is a cytokine which is used in certain therapies. The best expressed was an engineered version of the protein called NL-2/15. The cytokine was diffused into an assembled SpyCatcher-TRAP cage stabilized with 1,2-BBN. Analysis on denaturing acrylamide gels showed that the cargo was attached to the modified cage building blocks. In a key experiment the cages containing the NL-2/15 were added to modified HEK293T cells which can be used to monitor IL-2 signaling using colorimetric readout. When the cages were applied the cells and disassembled by UV light, IL-2 signaling was triggered. This showed that the NL-2 ligand liberated from the cage could exert its physiological effect. It is an exciting proof-of-principle experiment.

I have one question here. If I read the graph in Fig. 54B correctly, cells' response is also observed without UV light (although one order of magnitude weaker). Is this a problem for potential applications of similar systems, what is the mechanism behind it, and can it be prevented?

The Discussion section of the thesis is comprehensive and thoughtful and avoiding any overinterpretation or overselling of the results. During the defense, I would like the Izabela to speculate about the future developments of the cage technology. What are the key advantages of the TRAP-cages compared with other available designs, including recent artificial cages designed by the Baker group. What are the applications these cages could find in a near future? Are there any ideas as to how trigger disassembly by for example magnetic field or ultrasound? Which mode of delivery would be preferred? Extracellular disassembly or perhaps after internalization into the cell?

Overall, I rate the quality of the PhD student's research highly. She achieved the goal of generating cages that can be disassembled by a physical factor to release an active molecule which exerts a biological effect. This research is an important step toward fully functional cages that can be used for targeted and controlled drug delivery. The presented research is carefully designed and very thoughtful. Notably, Izabela is the first author a publication in the respectable Science Advances.

In summary, the dissertation presented to me for evaluation describes research at a high technical and scientific level. I believe that the dissertation meets the conditions set forth in the Act

on Scientific Degrees and Academic Title and on Degrees and Title in Art and with full conviction propose to admit Ms. Izabela Stupka, M.Sc. to further stages of the doctoral process. At the same time, taking into account the high quality of the dissertation, high level of the presented research, a very important field of research and the publication success, I propose to award the dissertation with an appropriate distinction.

Marcin Nowotny