Review of the doctoral thesis: **"Engineering of Protein Cages for Biomedical Applications"** by **Izabela Stupka**

Protein cages are emerging candidates in a diversity of biomedical applications, ranging from delivery vehicles, imaging tools to more advanced functions in, for example, enzyme replacement therapy. While the first studies in this direction mostly centered around protein cages obtained from a diversity of viruses, later also naturally occurring cages from other origin are studied and the field now naturally evolved to artificial / designed protein cages. The doctoral thesis of Izabela Stupka discusses the first steps in the employment of so-called TRAP protein cages – developed in the laboratory of Prof. Heddle – in biomedical applications. Towards this goal stabilization of the cage structure and triggered release are studied, together with more fundamental studies. These studies are timely and of high scientific value in the growing arena of bionanotechnology, furthermore, the thesis is of excellent quality – see further motivation below – as are the publications that (partly) find it origin in the dissertation. So I have no reservation that the thesis has the quality and contains sufficient quantity of work to admit the candidate to the defence.

Motivation (*questions and remarks in italics)*:
For a good thesis, it is important to first give context and sketch the state-of-the-art in the relevant field of research. Stupka gives in her introductory section first some background on compartmentalization in Nature and the role protein cages play. Next, the structuring of protein cages is discussed, followed by ways of engineering them. To further control properties, the subsequent part is dedicated to artificial protein cages, which gives an appropriate overview of the state-of-the-art. (*On page 22, Fig. 5, the size and mentioned diameter of CCMV is wrong 18 nm vs. 28 nm.*) Different strategies for cage design are listed, as well as strategies to stabilize the protein assembly and to encapsulate foreign cargo. This section is concluded with a brief overview of medically relevant applications of protein cages. *In the "Encapsulation of Protein Cages" section could certainly do with some CCVM based examples. The examples chosen seem to be a bit randomly selected.*
The introductory section is good and serves its purpose. The formulated "Aims of the Research" at p. 40 are clear and concise.

The "Materials and Methods" section is accurate and has sufficient detail to allow reproducing of the data. *No further comments.*

In the first part of the Results section the application of different cross linkers is described in order to stabilize the TRAP cages. With a variety of techniques, mostly PAGE, TEM and LS, the structure and stability of the formed cages is studies. It is concluded that cages first have to be templated with gold, where the crosslinker can replace the gold atoms to stabilize the protein cage. In general the data, convincingly, points to cage structures with 24 TRAP rings connected by covalent bonds. In the second part, the stabilized cages are loaded with other protein cargo by genetic fusing it to the N-terminus of the TRAP. This packaging was successful with a chosen FRET pair that apart from encapsulation, also allowed for monitoring the disassembly kinetics of the cages.
Upon successful crosslinking of cargo equipped TRAP rings, the next step was to use photocleavable linkers. This was successfully shown for the, so-called, BNN cross-linkers (*I am not sure about the mechanism in Fig. 40 in the first and second step. Does DDT react with*

the aldehyde or with the Cys thiol?). Also for this linker system, cargo encapsulation was studied, albeit with a low yield.
In the final results section, a model system with the HEK-Bleu IL-2 cell assay was used to give a first hint on biomedical applicability of the photocleavable TRAP protein cage.

In the thesis a separate discussion section is chosen, which not always furthers the readability. Nevertheless, the section is clear and addresses the majority of the questions that arise from the data. Furthermore, it places the experimental work in the proper context – also in relation to existing literature. In my opinion the discussion is adequate and of a high standard! Some minor questions remain (for example: *why does the SpyTag-SpyCatcher system have lower yields that the FRET example*), but evidently steps towards real applications have been made and certainly a better understanding of this versatile protein cage has been gained.

The perspectives given by the author are realistic and do not raise unrealistic expectations. Clearly, challenges such as the triggering of the immune response need to be overcome, but the presented work indeed broadened the spectrum of possible utilization of the TRAP protein cage system.

Oct. 31, 2022
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