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Opinion on the PhD thesis by Mantu Kumar

Mantu Kumar has presented a PhD thesis entitled "Design and production of highly programmable DNA protein cage hybrid nanostructures". Overall, the aim of the thesis is the generation of macromolecular cages that can be controlled by small molecules. The thesis can be divided into two subprojects, a "safe" first project, and a more adventurous "risky" second project that yielded the most interesting results in this thesis. Both parts are published, in the journals Nanoscale and Nanoletters. For the Nanoscale paper with the results from the first part of the project, Mantu Kumar is the first author. For the other paper, he is the third author. This latter paper has generated a lot of interest in the community, as evidenced by 74 citations in the five years since publication according to google scholar. Overall, Mantu Kumar's publication record with 122 citations in total is very good for this career stage.

The first part of the thesis builds on prior work for a ferritin from the archaebacterium *Archeoglobus fulgidus*, termed AfFtn. From the prior work, it was already known that the protein can form dimers, which can be triggered to assemble into cages by the addition of monovalent and divalent cations. It was also known that the orthologue of AfFtn from the hyperthermophile *Thermotoga maritima*, TmFtn, also forms dimeric assemblies and cages. Remarkably, the symmetry of the cages differs: although both proteins form 24-mers, the TmFtn cages are octahedral, whereas the AfFtn cages are tetrahedral. This structural change is all the more remarkable, since the sequence identity between AfFtn and TmFtn is very high (51% identity). Can the change of symmetry of the cages be explained by inspection and comparison of the structures? For AfFtn, it was already known that assembly could be triggered by Mg^{2+} binding, and Mantu Kumar could show that this feature was shared with TmFtn. Inspection of the structures suggested a mechanism for reversible cage formation. The structures showed that cage assembly brought a conserved glutamate (E65) into proximity to other acidic residues. This juxtaposition should be unfavorable in the absence of cations, but might be favorable if the acidic residues served as ligands for a metal cation. If this explanation for reversible cage formation was correct, then replacement of the glutamate (E65) by an aspartate should have little effect. By contrast, replacement of the glutamate by a basic residue (arginine or lysine) should make the interaction constitutively favorable, irrespective of the presence of metal cations. This is indeed observed, as summarized on page 51. There is a small surprise though: assembly remains Mg^{2+} dependent for the E65Q variant of the protein, but is only "minimally metal ion dependent" for the E65A variant of the protein. This difference is somewhat puzzling to me: both the E65Q and E65A substitutions abolish the side chain charge of the glutamate. Why does mutation to alanine have a

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more drastic effect?

The second project starts from an interesting observation. At one extreme of the spectrum, protein cages are good at sequestering small molecules or metabolites, with relatively little leakage. However, since they are typically highly symmetric multimers, the possibilities to vary cage surface are subject to severe symmetry constraints. At the other extreme of the spectrum, cages that are built from DNA origami offer almost limitless possibility for modifications, including asymmetric modifications, but are typically leaky. This suggested to Mantu Kumar and his colleagues to develop a hybrid design that brings together the best of both worlds, by packaging a protein cage into a DNA origami box, termed a glove. Connection between the protein cage and the origami glove is made by derivatizing lysine residues of the protein cage with single stranded DNA, which can hybridize with single strands of DNA emanating from the glove. The chosen derivatization chemistry is a two-step process. First, the primary amines in lysine side chains are made to react with the N-hydroxysuccinimidyl portion of a DBCO-sulfo-NHS linker. Subsequently, the dibenzocyclooctyne portion is reacted, presumably in a copper-free click reaction, with ssDNA carrying an azide label. By design, the glove was supposed to adopt the shape of an open box ("bathtub"), which should be large enough to accommodate the protein cage inside. The origami design of the glove was apparently not straightforward. According to Mantu Kumar's description, AFM images showed that two of the four walls were missing. For me, this would have been difficult to deduce from the AFM image in Fig. 22 of the thesis. Was there further image processing involved to confirm this conclusion? Also, was the information specifically useful to improve the design, or was the second design simply another, "luckier" attempt? For my own education, I would be curious to learn how iterative origami design is done, based on a characterization of results from an earlier design. Mantu Kumar and colleagues had a surprise when they analyzed the outcome of their final design. Instead of protein cages inside the glove, they found protein cages above the glove. It is unclear to me though whether this form of functionalization would interfere with planned applications. If it does, could shorter DNA bridges between the protein cage and the origami glove solve the problem? In all attempts, protein cages inside gloves were never the only species. Could this be solved by subsequent affinity purifications for a protein tag, and the presumably acidic DNA origami glove (which should bind strongly to cation exchangers)?

Overall, the thesis is traditionally structured, except for the reasonable division of the work into the two separate projects. The thesis is concise and editorially at a high standard, and has an extensive bibliography. While I am happy with the very concise presentation of the work in general, I would have wished for a more substantive discussion.

The strong side of the thesis is the diversity of methods that have been used in the project. These include protein production, generation of protein variants, DNA origami design, spectroscopic methods, X-ray crystallography, transmission electron microscopy (TEM), and atomic force microscopy (TEM). Some of the methods are well documented, with a good amount of raw data about

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mass spectrometry, chromatography and protein purity, as well as DNA origami fragments. The crystallographic table indicates that the refinement of crystal structures has been well done. Not being an expert on DNA origami, I would struggle to replicate the design of the origami container/glove, even though key tools (cadnano, CanDo) are indicated. In fairness to Mantu Kumar, this is understandable, since the actual design appears to have been done by a collaborator (Dr. Yusuke Sakai). Also, to Mantu Kumar's credit, the result itself should be reproducible for others, since the staples used for DNA glove production are all listed in the Appendix. Just the design process to arrive at the listed sequences remains somewhat unclear. For my own education, I would like to learn more about the design process at the thesis defense.

The weaker side of the thesis is the lack of clarity about a concrete biological application. It is of course understandable that an actual demonstration would be beyond the scope of this materials science thesis. However, I do feel that more effort could have been made to lay out at the least the ideas in the Discussion. For the first part of the thesis, I understand that the constitutively cage-like TmFtn was primarily made to demonstrate the mechanism of reversible cage-formation of the wild-type protein that is considered for application. However, it remains unclear to me how Mg^{2+} control of cage assembly could be used *in vivo*. The mechanism for Mg^{2+} controlled cage formation suggests that other cations could substitute for Mg^{2+}. Concentrations of Na^+ and K^+ are high in the extracellular and intracellular milieu, respectively (of the order of 150 and 300 mM). Is the Mg^{2+} selectivity sufficient that these ions would not dominate as the determining factor of the assembly state? If it is, could changes in Mg^{2+} concentration between different compartments be exploited for control of the cage and non-cage forms of the proteins? I have seen intracellular Mg^{2+} concentration quoted as between 10 and 20 mM, and free Mg^{2+} concentration as between 0.5 and 1 mM. Assuming that free Mg^{2+} matters, this would suggest that cages could be closed in blood, and then open in an intracellular milieu? Would the Kd for Mg^{2+} be in the correct range? And how would the cages be imported into cells? While the nuclear membrane has huge pores (assembled 26S proteasomes can pass), the cytoplasmic membrane does not. How would cages get into cells? For the second part of the thesis, I would have liked to see a clear concept for the use of the gloved cages in the Discussion. As the paper on the gloved cages has already been cited 74 times, there would have been a great opportunity to discuss what other developments have since been triggered by the work. If patent protection rules are not in the way, plans for applications could be made clearer in the discussion at the defense.

Despite my minor reservations that are mostly due to my perspective as a molecular/structural biologist and not a materials scientist, I have no doubts that the thesis fulfils the conditions laid out in article 187 of the law of July 20th 2018 that regulates scientific higher education and I therefore recommend to the Scientific Council of Jagiellonian University to proceed with the next steps to award Mantu Kumar a PhD degree.

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With best regards

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