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Review report on the Doctoral thesis of MSc Mantu Kumar entitled "*Design and production of highly programmable DNA-Protein cage hybrid nanostructures*"

The dissertation of Mantu Kumar entitled "*Design and production of highly programmable DNA-Protein cage hybrid nanostructures*" was prepared under the supervision of Professor Jonathan Heddle in the Bionanoscience and Biochemistry Laboratory, Malopolska Centre of Biotechnology, Jagiellonian University.

The thesis concerns the issue of formulation of macromolecular cages based on ferritin derived from a hyperthermophile bacterium (*Thermotoga maritima*, TmFtn) that are capable of encapsulating cargo molecules, for instance, drug compounds, in a leakage-free manner. In particular, Mantu Kumar has tried to solve the issue of efficient and controlled disassembling and assembling of the protein cage in mild conditions without disrupting the function and structure of ferritin and the decomposition of cargo molecules. Indeed, the development of such macromolecular cages for encapsulating pharmacologically active compounds is significant from the industrial and medical points of view. In the first part of the thesis, Mantu Kumar engineered the dimeric interface of TmFtn to understand the mechanism of the divalent metal cation-dependent assembly of the cage and modulate its formation. To understand the mechanisms of a cage assembly, he generated a series of ferritin variants and applied numerous biophysical techniques, including X-ray crystallography, TEM imaging, analytical SEC, native PAGE, circular dichroism, mass spectrometry, dynamic light scattering, inflection temperature and differential scanning calorimetry measurements as well as bioinformatics analysis. In the second part of the thesis, the author has created a hybrid structure composed of DNA origami open container (easily programmable in size, shape and selectivity, however leaky for a cargo), which selectively binds a single ssDNA-surface modified engineered TmFtn molecule (leakage-free for a cargo compound but limited in modifications). Within this

study, the author applied bioinformatics tools to design a DNA origami container that is specifically capable of interacting with a modified ferritin cage. Next, he produced the container and ssDNA surface-modified TmFtn, which were used to generate a DNA-protein cage hybrid nanostructure. To analyze biophysically the container, modified nanocage and the resultant hybrid material, Mantu Kumar applied, among others, gel-based assays, atomic force (AFM) and transmission electron (TEM) microscopies.

The presented work was supported by several external sources, including grants from the *Foundation of Polish Science* and *National Science Centre*, led by the promotor of the Ph.D. candidate. It is worth noting that the thesis outcomes were published in 2019 and 2021 as two research articles in leading journals, namely *Nano Letters (ACS)* and *Nanoscale (RSC).* The layout of Mantu Kumar's doctoral dissertation is classic. The text of the work, 104 pages long, is divided into an abstract, a literature part, aims of the work, a description of materials and methods used, the results of his research (divided into two parts) with a short discussion section, lists of figures and tables and the appendix containing details of experiments performed within described studies. The purpose of the work is formulated correctly. The literature review is supported by numerous references published in recognized periodicals. This section briefly and sufficiently describes the current state of knowledge regarding the research objects, including natural and artificial macromolecular cages, DNA origami and DNA-protein hybrid nanostructures, as well as their application and challenges that must be overcome. It introduces the reader very well to the presented and is based on correctly selected and current literature. The approach to setting the main goals, as well as the description of the results and discussion of the work, do not raise my significant reservations. They prove the Ph.D. student's excellent substantive preparation for scientific work. Notably, the obtained results required the application of numerous methods from the intersection of bioinformatics, molecular biology and biophysics. In my opinion, the most significant achievements include:

1. An engineering of the wild-type TmFtn (that requires a high concentration of Mg^{2+} cations to assemble) to obtain different mechanisms of cage assembly and stability. Based on structural analysis of the native ferritin, Mantu Kumar generated several singe substitution (E65) variants of the ferritin cage. Among them, E65A, E65Q and E65D variants form the cage at much lower concentrations of magnesium ions in comparison to the wild-type TmFtn. Two other substitutions with basic amino acids (E65K and E65R) make the cage assembly in an Mg^{2+}-independent manner. Crystallographic studies explained different mechanisms of cage formation for all variants under investigation. In addition, all variants

were characterized biophysically in a detailed manner. Based on performed studies, E65R variant was selected as a cargo-carrying component of DNA-protein cage hybrid nanodevice.

2. Preparation and characterization of the hybrid nanodevice composed of an open DNA origami container and ssDNA-anchored E65R variant of TnFtm. Within this nanostructure, the interactions between the protein cage and container are based on base-pairing between ssDNA attached to the TmFtn surface and the DNA origami's complementary sequence. Such interactions ensure a high conjugation. One of the most significant advantages of the obtained device is the introduction of asymmetry into the cage molecule, as only a part of the ferritin surface is exposed to the solvent region. In turn, the exposed surface of the cage can be selectively modified for particular purposes.

Considering the above, I rate the whole work very highly, which does not change the fact that as a reviewer of the thesis, I have a few minor comments and a question which I listed below:

1. From the editorial point of view, the thesis is well organized, as well as carefully and well written. I found only a few typing errors or inconsistencies within the text, which are listed below:
    - In the "Materials and Methods" section, the procedure for attaching ssDNA to ferritin is omitted.
    - Throughout the "Materials and Methods" section: mL or μL not ml or μl for milliliters or microliters is the current abbreviation
    - Page 23, "Gene sequencing" subsection: "The gene sequence chromatogram…", should be "electrochromatogram".
    - Page 43, "*Crystal structure of TmFtn*" subsection: 'Crystal structures were solved with the help of program Phenix …' should be "Crystal structures were solved with the help of program Phaser from the Phenix package".
    - Page 45, Figure 4D: The panel should present coordinated cation, not elemental iron. The analogous concern relates to using Mg instead of Mg^{2+} (Page 72).
2. Other comments or questions:
    - It is mentioned (Page 48) that an electron density corresponding to a fatty acid is observed between ferritin dimers. Is the fatty acid exposed to the solvent area and, in turn, affect the interaction between ssDNA-anchored TmFtn and DNA glove?
    - Table 3 (Pages 46 and 47): atomic displacement parameters (ADPs, formerly B-factors) are high or very high for protein atoms (~50-86 $Å^2$) and especially for

ligand atoms (76-107 $Å^2$). Could it be explained in any way? Since the refinement procedure of described crystal structures is limited, it is hard to speculate a reason for high ADPs within this review. Data processing statistics in Table 3 suggest that the authors overestimated some data resolutions (signal-to-noise ratio below 1 and CC1/2 less than 50%). Please comment on this issue.

- Figure 13 (Page 49). There is no side chain for residue D134 in some panels. Is this residue disordered in some ferritin variants or intentionally omitted due to a lack of interaction with residue 65?
- It is mentioned (Page 72) that Mg^{2+} ions were not identified in the crystal structures of the WT TmFtn cage. Indeed, confirmation of a site that is occupied by Mg^{2+} cation might be tricky. Was it considered to soak or co-crystallized crystals of WT-TmFtn with heavier divalent metal cations, for instance, Ca^{2+}, Mn^{2+}, Sr^{2+} or Ba^{2+}? These cations are readily detectable in native and/or anomalous electron density maps.

Despite some minor critical remarks and comments, I have a favorable opinion of the doctoral thesis of the Ph.D. candidate. The studies performed and presented by Mantu Kumar are ambitious and require the extraordinary skills of a doctoral student. Hence, I believe that the work submitted for assessment meets all the requirements for the doctoral thesis. Therefore, I recommend to the Scientific Council for Biological Sciences of the Jagiellonian University to admit Mantu Kumar to further steps of the Ph.D. procedure.

Reasumując, rozprawa doktorska mgr Mantu Kumara przedstawia bardzo interesujące wyniki dotyczące projektowania i tworzenia klatek makromolekularnych. Biorąc pod uwagę wysoki poziom naukowy pracy, jej interdyscyplinarny charakter oraz zakres świadczący o samodzielności Doktoranta w prowadzeniu badań naukowych, stwierdzam, że rozprawa spełnia kryteria określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r. poz. 1668 z późn. zm.). Dlatego zwracam się do Rady Dyscypliny Nauk biologicznych Uniwersytetu Jagiellońskiego o dopuszczenie mgr Mantu Kumara do dalszych etapów postępowania o nadanie stopnia doktora w dziedzinie nauk ścisłych i przyrodniczych w dyscyplinie nauki biologiczne.

Krzysztof Brzeziński