**Official opponent's REVIEW on the dissertation for the degree of Candidate of Biological Sciences written by Wang Yueqi on the topic: "Characteristics of the Structural Organization and Interaction of Tailed Bacteriophages with Biofilm-Forming Bacteria"**
**in Biophysics (1.5.2 – "Biophysics")**

The presented thesis has a standard structure including introduction, literature review, description of materials and research methodology, the results section with the elements of discussion, the discussion part itself, outlined conclusions, acknowledgement section, list of author publications covering the research topics of the thesis, reference list containing 124 entries and three supplementary tables.

Introductive part contains all necessary positions, with especially well formulated central aim and specific tasks. Novelty and relevance statements are also included. The motivation of the entire study is prominently stated and includes practical biomedical aspects of adopting phages to counter antibiotic resistant bacterial strains, especially those responsible for intra-hospital infections. Thus, all formal criteria are met.

Tailed phages, especially "giant" species, recently attracted a lot of attention, especially due to the peculiarities of their replication. Sometimes they are even considered, along with eukaryotic giant DNA viruses, as the precursors of the eukaryotic nucleus, although these theories are marginal.

The shift of focus towards phage therapy of bacterial diseases is stimulated by growing number of antibiotic-resistant strains, largely caused by uncontrolled use of antibiotics at the cusp of centuries, and due to the stagnation in developing novel pharmacological anti-microbial agents. On the other hand, phages co-evolved with bacteria for billions of years in a mutual arms-race and both survived. The adaptability and "evolvobility" of phages make them really good and possibly the only candidate for developing future tools for bacterial diseases control.

The review spanning 21 pages is well written and easy to read. It introduces to the reader the wonderful world of phages, briefly describes the history of phages' applications as antibacterial agents and delves into peculiarities of phage-biofilm

interactions. It somehow shifted my view on the phages. Although some recent papers present them as biological nano-robots that can even walk over bacterial surfaces using Brownian ratchet mechanism in search for the most curved membranes, the review emphasizes the presence of a staggering array of built-in enzymes, facilitating phages navigation and homing, although in a “passive mode”, without active force generation and energy consumption.

Of minor note, part 3.5.2 leaves a strange feeling, describing phages exploiting biofilm’s water channels, that are “size-restrictive for large antimicrobial agents” and “phages exploiting their small size” to pass these barriers. And the conclusion is that a multimegadalton phage penetrates the biofilm more efficiently than an antibiotic of several kilodaltons at large. It is even more confusing, considering the focus of the work on the giant phages. Either it is a bit of a mess, or it should be considered along with the phages’ enzymatic arsenal, described in the text above.

The part about bacterial persisters was totally new to me personally, brief, but very interesting.

The second part of review deals with the experimental toolkit for studying phages morphology and virus-bacteria interactions. This part, although written as good as the first part, seems to be a little odd. All of the described methods are well established and do not require such an introduction apart from the detailed description in the methods part and in the results section in the form of “to do so we employed TEM, and to prove this we used kill-time assay” and so on.

What seems to be missing is the more or less detailed description of the phages under study and the rationale for this particular choice.

Materials and methods sections are over-detailed. A surprisingly massive part dealing with cryo-EM structure determination of phage’s head portal vertex seems to contain data belonging to the results section, especially the images, and perhaps the description of particle classification strategy and reconstruction. This note also applies to part 4.3.5, AlphaFold modelling, and to less extent, 4.3.5, Molecular refining, which, again, follow the style of results descriptions or even the discussion section, rather than that of “Experimental procedures”. This is, however, a minor formal shortcoming.

It should be noted that the scale bars are missing on figures 7-12. This is a persistent and recurring problem.

In part 4.4 “Scaffold preparation” molar ratio of the solvent components is given without providing exact concentration.

The first part of the Results section continues the story, started in Materials and methods, about the structural reconstruction of multimolecular assembly, that connects the head of TaPaz phage to its contractile tail and contains a portal complex that arranges the traffic of phages genomic DNA into the head assembly during maturation of the virion and out of the head at the initiation of infection.

The complete assembly is about 25 nm in diameter and nearly 45 nm long was reconstructed with resolution less than 0,32 nm, that is impressive result for such a complex structure.

Overall, this part of the study is a very impressive structural biology research, but it is not covered by the author’s papers listed. Perhaps it is pending for publication. But here I should note that the legend for fig. 13, that depicts the final model, contains reference to Wang et al., 2022. This work is mentioned neither in references, nor in the list of papers prepared on the basis of the presented thesis.

The second part of the results section switches attention to the other phage, phiKZ, and its interaction with the bacterial host during the infection process. The primary finding of this study is the detailed description of the membrane-bound vesicles characteristic to the early stages of infection, and containing phage’s genome and early infection proteins. The presented data hint at the outright peculiar mechanism of phage-driven bacterial membrane invagination and vesicle fission. May this mechanism point on some evolutionary steps preceding formation of eucaryotic endomembranes or the endocytosis?

These findings also provide even more complicated questions about how membrane-enclosed phage genome interacts with bacterial metabolism at these early stages of the infection? Very interesting.

Figure 20, lower panel. White arrow designating bacterial nucleoid points onto nothing. It might be better not to place it at all. However, this picture is a “citation” from ref. 113, which seems not quite appropriate for the Results section, while the author’s own papers contain enough material for this illustration.

It is not completely clear neither from the text nor from the article on the subject wherever the details of stage 3 of the presented three-stage model of phage infections were observed directly? Especially it concerns the EPI vesicles docking to the “nucleus”.

The third and fourth parts form the core of the study. The third part deals with the structure of the biofilms formed by various bacterial strains, including notorious and highly resilient hospital isolates of *Pseudomonas aeruginosa*. The primary research instruments here are scanning electron microscopy and classic and robust microbiological toolkit.

The primary results of this part seem to be the following - the morphology and robustness of biofilms depend on both genetic features of particular bacterial strain and environmental factors, modelled in this study by the culture medium variations and pH/salinity modulation, while the susceptibility of biofilms to the phage attack depends on its robustness and the presence of matrix polysaccharides. This outcome opens a possibility to fine-tune the phage-based treatment to each particular case.

Although presented data are convincing, this part, concerning phage-biofilms interaction would greatly benefit from the presentation of TEM micrograph of the phage-treated biofilms to complement SEM data with data on the internal structure of bacterial cells and the progress of the phage infection.

This part of the text is exceptionally wordy and hard to read. A bit of direct citation: “Scanning electron microscopy revealed sequential architectural transformations during phage exposure. Initial low-magnification views depicted dense, planar biofilm matrices, while high-resolution examination showed characteristically loose cellular arrangements with substantial intercellular voids. After two hours of phage exposure, macrostructural integrity remained intact, though high-magnification

imaging detected incipient cellular deformation - PhiKZ-treated samples developed fibrous EPS, whereas 14/1-exposed biofilms exhibited increased cellular packing density with minimal EPS production . By four hours, both phage treatments induced extensive crack networks resembling jigsaw puzzles at low magnification, signaling structural compromise, while high-resolution views confirmed enhanced EPS secretion and progressive cellular dispersion".

Maybe it would benefit from the introduction of formal criteria for describing the biofilms' morphology and to replace such narrative descriptions, which was introduced in the author's abstract as the percentage of the residual biofilm after the treatment.

Fig. 24 and 25. Scale bars are mentioned, but are missing on the micrograph or are lost in the unreadable faint text at the bottom of the figure panels. This notion also applies to fig. 36 and 47-48.

The final part of the results section is devoted to the development of a carrier matrix that can absorb phage particles and provide their prolonged release for therapeutic applications.

The puzzling question for this part is the following: why silk fibroin in particular and why protein in general for wound- and burns-treatment applications? In this case foreign - insect - proteins come in contact with the immune cells populating the wound and this contact can result in an unwanted acute immune response.

Nonetheless, the presented results confirm the capabilities of the designed carrier - the composition of silk fibroin augmented with polyethyleneimine. It showed the best results in absorbing phage particles, dynamics of their release and, low toxicity towards eukaryotic cells, biodegradability, and, as a bonus, appeared to be bactericidal by itself, thus supporting the antibacterial activity of the phage. Of note, this part of the study didn't involve carrier tests on bacterial biofilms.

Overall impression from the results section is that the study is chimeric, loosely knit from several marginally related fragments, each of which is largely self-sufficient. I was totally unable to trace the link between TaPaz phage neck complex structural biology and silk-fibroin-PEI-phage combo as the anti-biofilm pharmaceutical

combination, but it doesn’t compromise the significance of each part of the presented research by itself.

The discussion section is nicely short and starts with the perspectives and challenges of phage-based therapy, but soon after abruptly switches to the recapitulation of the results, which is very common problem in students’ diplomas and Ph.D. students' thesis, promoting the increasing tendency of merging results and discussion into one joined section.

Three final paragraphs provide a sketchy description of perspectives of the study based mainly on the results concerning phage-biofilm interactions and phage carrier with controlled release, finally mentioning possible adverse effects of possible patient’s immune and inflammatory response.

Conclusions and Findings sections repeat each other and most of the Discussion section.

The thesis also contains three supplemental tables with the results of analysis of the interactions between three protein substructures of TaPaz phage head-to-tail connector.

The results of the study were presented in 4 papers, each recapitulating corresponding Results section, except the structural biology part.

Overall impression of the text is very satisfactory, despite the above-mentioned wordiness, peculiar miss-wordings, which are sometimes very confusing and a couple of missing prepositions and footnotes.

In conclusion, the presented thesis corresponds to specialty 1.5.2 – “Biophysics” in the following areas: "Supramolecular assemblies. Metastable states and mechanisms of self-organization of biomolecules in vivo and in vitro" and "Medical biophysics. Aviation, space and marine medicine. Clinical immunology and laboratory diagnostics. Regenerative medicine. Pathologies associated with disruption of biophysical processes", as well as specialty 1.1.10 – Biomechanics and bioengineering in the area of "Creation of organ and tissue substitutes, biomechanical instruments and devices".

The above-mentioned shortcomings do not affect the significance and the validity of this dissertation. The dissertation meets the requirements established by Lomonosov Moscow State University for works of this kind. Its content complies with the specialties 1.5.2 – "Biophysics" and 1.1.10 – "Biomechanics and Bioengineering" (in the biological sciences), as well as the criteria defined in paragraphs 2.1-2.5 of the Regulations on Awarding Academic Degrees at Lomonosov Moscow State University. The dissertation is formatted in accordance with the requirements of the Regulations on the Dissertation Defense Council for Candidate of Sciences and Doctor of Sciences Degrees of Lomonosov Moscow State University. Thus, applicant Wang Yueqi deserves to be awarded the academic degree of Candidate of Biological Sciences in the specialty 1.5.2 – "Biophysics" and 1.1.10 – "Biomechanics and Bioengineering".

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