ABSTRACT

dissertation for the degree of Doctor of Philosophy (PhD) in the educational program "8D05105-Biotechnology"

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on the topic: **"Production and characterization of nanoantibodies against rabies virus"**

General description of the dissertation. The dissertation focuses on the development of novel biological agents against the rabies virus. During the study, a recombinant trimeric form of the rabies virus glycoprotein was generated based on the trimerization domain of human collagen type XVIII, and its immunogenic and protective properties were determined. Using phage display technology, nanobodies specific to the rabies virus glycoprotein were obtained, and various multimeric forms of these nanobodies were created.

Relevance of the research topic. Rabies is a highly dangerous viral infection that affects the central nervous system. Worldwide, rabies is endemic in more than 150 countries and territories. Each year, the disease causes approximately USD 8.6 billion in economic losses and claims more than 60,000 lives, with 96% of deaths occurring in Asia and Africa. The danger of rabies is associated with the current lack of effective treatment and the fatal outcome of the disease.

Rabies in humans also poses a significant threat to public health in the Republic of Kazakhstan. According to the Ministry of Healthcare of the Republic of Kazakhstan, three fatal cases of rabies were registered in the country in 2025, whereas no such cases were reported in 2024. In 2025, 60,153 people sought medical care due to animal bites, scratches, or exposure to animal saliva, while 14,134 people sought medical attention during the first quarter of 2026. Natural foci of rabies persist in Kazakhstan, and cases of the disease continue to be reported among animals. Therefore, improving rabies prevention measures and developing effective biological products against this infection remain important and relevant objectives.

Specific prophylaxis of rabies is based on the use of vaccines and rabies immunoglobulins. However, the risk of adverse reactions, dependence on donor-derived material, high cost, and limited availability restrict the widespread use of conventional immunoglobulins. Therefore, the World Health Organization recommends the development of alternative biological products for rabies prevention.

Nanobodies are considered promising biological agents against viral infections due to their high specificity, stability, solubility, and ability to penetrate tissues efficiently. The possibility of engineering nanobodies in various formats using genetic engineering approaches allows their functional properties to be improved. In particular, multimerization may enhance their avidity and virus-neutralizing activity. Therefore, the development of highly specific nanobodies against the rabies virus and the investigation of their protective activity represent a relevant area of scientific research.

In this regard, the development of novel nanobodies targeting the rabies virus G glycoprotein, as well as the enhancement of their specific binding and protective activity, represents one of the relevant approaches to improving biological products against infectious diseases.

Objects of study: recombinant proteins rRABV-GE and rRABV-G-XVIII, as well as recombinant nanobodies specific to the rabies virus glycoprotein that possess virus-neutralizing activity.

Subject of study: the immunogenic properties of the trimeric form of the rabies virus glycoprotein, as well as the specificity, affinity, and virus-neutralizing activity of nanobodies.

The aim of the study is to construct a recombinant trimeric form of the rabies virus glycoprotein (RABV-G) based on the trimerization domain of human collagen type XVIII and to evaluate its efficacy in vitro and in vivo, as well as to develop novel nanobodies for the effective neutralization of the rabies virus and assess their biological activity**.**

Research objectives:

1. Construct recombinant genes encoding the monomeric (rRABV-GE) and trimeric (rRABV-G-XVIII) forms of the rabies virus glycoprotein (RABV-G) ectodomain.
2. Achieve functional expression of rRABV-GE and rRABV-G-XVIII proteins in an *E. Coli* system and isolate the recombinant proteins in purified form.
3. Evaluate the immunogenicity and antiviral protective activity of rRABV-GE and rRABV-G-XVIII proteins *in vitro* and *in vivo*.
4. Construct a cDNA library of nanobodies (VHH) specific to the recombinant RABV-G-XVIII protein using phage display technology and select virus-specific phage clones.
5. Generate di- and trimeric nanobodies specific to the recombinant RABV-G-XVIII protein.
6. Characterize the mono-, di-, and trimeric nanobodies and evaluate their antiviral protective activity *in vitro* and *in vivo*.

Research methods: molecular-genetic methods (gene isolation and cloning, RT-PCR, PCR), biochemical methods (affinity and gel-filtration chromatography), immunological methods (ELISA, Western blotting, virus neutralization assay), as well as phage display methods (biopanning) and gel electrophoresis.

Scientific novelty of the study:

- A trimeric recombinant form of the rabies virus glycoprotein, rRABV-G-XVIII, was generated for the first time using the trimerization domain of human collagen type XVIII; the protein was expressed in the *E. coli* SHuffle system and purified to a high degree.
- The rRABV-G-XVIII protein demonstrated high immunogenic and protective properties. When administered without an adjuvant, rRABV-G-XVIII induced a virus-specific antibody response in mice and conferred complete protection against a lethal dose of rabies virus.

- Specific anti-rabies virus nanobodies were isolated for the first time from a cDNA library of dromedary camels immunized with the RABV-G-XVIII antigen, and their dimeric and trimeric forms were synthesized. A comparative assessment of the biological activity of monomeric, dimeric, and trimeric nanobodies revealed that the trimeric form provides 100% protection against a lethal dose of rabies virus *in vivo*.

Practical significance of the study. The research findings establish a scientific and practical foundation for developing next-generation recombinant rabies vaccines, therapeutic nanoantibodies, and highly sensitive diagnostic test systems. The engineered recombinant antigen rRABV-G-XVIII is considered a promising candidate for the development of effective subunit rabies vaccines. The high in vivo protective activity of the trimeric nanoantibodies suggests their potential use as agents for post-exposure prophylaxis and the treatment of rabies.

Theoretical significance: This study contributes to a deeper understanding of the relationship between the structural organization of the rabies virus glycoprotein (RABV-G) and its immunogenic properties. The results confirm the important role of the antigen's trimeric conformation in the generation and enhancement of the immune response, thereby expanding theoretical knowledge in the fields of structural immunology and virology. Furthermore, a comparative study of monomeric, dimeric, and trimeric forms of nanobodies derived from dromedary camels provides new scientific data on the impact of nanobody multimerization on their biological activity and offers deeper insight into the mechanisms of virus-neutralizing action.

Key findings presented for the dissertation defense:

1. The rRABV-G-XVIII construct, fused with the trimerization domain of human collagen type XVIII, was expressed for the first time in the *E. coli* SHuffle expression system and isolated in purified form.
2. The RABV-G-XVIII antigen was shown to possess higher immunogenicity and efficacy compared to the recombinant rRABV-GE protein. It was established that RABV-G-XVIII forms a stable trimeric structure, induces a robust humoral immune response, and exhibits virus-neutralizing activity comparable to that of an inactivated rabies vaccine.
3. Administration of the RABV-G-XVIII antigen without an adjuvant induces high levels of high-affinity virus-neutralizing antibodies and provides complete protection for all experimental animals against a lethal dose of the rabies virus. These data demonstrate the high stability of the trimeric protein expressed in the bacterial system and its potential for the development of an effective and affordable vaccine.
4. Anti-rabies virus nanobodies were generated and characterized using phage display technology from a cDNA library of dromedary camels *(Camelus dromedarius*) immunized with the RABV-G-XVIII antigen.
5. To enhance the virus-neutralizing activity of the nanobodies, dimeric and trimeric forms were engineered, resulting in a significant increase in their affinity for the virus.

6. A comparative study of monomeric, dimeric, and trimeric nanobodies revealed that the trimeric form provides 100% protection against a lethal dose of the rabies virus *in vivo*. This confirms the high therapeutic potential of these molecules.

Alignment with the research plan. This dissertation was conducted within the framework of the grant project AP14870256 (2022–2024) funded by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan, titled "Development of novel nanobodies for the effective neutralization of the rabies virus and their application as highly specific and sensitive reagents for rapid diagnostics."

The doctoral candidate's personal contribution to the preparation of each publication includes a review of the scientific literature, the planning and conduct of experiments, data collection, interpretation and statistical analysis, as well as the description and subsequent discussion of the results.

Validation of the research work. The findings of the dissertation were presented and discussed at the following scientific conferences:

1. International Scientific and Practical Conference "Genetics and Physiology: Past, Present, Future" (May 27–30, 2025, Almaty, Kazakhstan);
2. International Scientific Conference of Students and Young Scientists "Farabi Alemi" (April 3–5, 2025, Almaty, Kazakhstan);
3. 2nd International Conference on Biotechnology and Biomedical Research (April 9–10, 2025, Manila, Philippines);
4. VII International Scientific and Practical Conference "Prospects for the Formation and Development of a Scientific School of Pharmacy: Intergenerational Continuity," held as part of the ANaMed Forum – New Generation 2025 and dedicated to the 80th anniversary of Professor R. Dilbarkhanov (June 4–5, 2025, Almaty, Kazakhstan).

Publications:

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