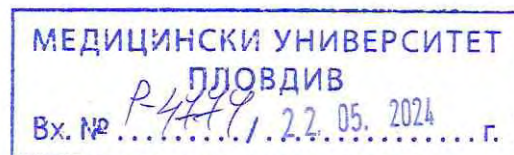


EVALUATION



from

**Assoc. Prof. Maya Margaritova Zaharieva – Stephan Angeloff Institute of Microbiology,
Bulgarian Academy of Sciences**

of a PhD thesis for awarding the educational and scientific degree 'doctor'

Professional direction 7.3. "Pharmacy"

PhD program „*Technology of pharmaceutical forms and biopharmaceutics*“

Author: *Nikolay Stantchev Zahariev*

PhD study: full-time PhD

Department: „Pharmaceutical sciences“. Faculty of Pharmacy, Medical University - Plovdiv

Title: *"Nano carriers for targeted drug delivery in antitumor therapy"*

Supervisor: *Assoc. Prof. Bissera Assenova Pilicheva, PhD, Medical University of Plovdiv*

1. General presentation of the procedure and the PhD student

The presented set of materials on paper/electronic media is in accordance with Art. 70 (1) of Section I. Acquisition of educational and scientific degree "DOCTOR" and scientific degree "DOCTOR OF SCIENCES" at MU-Plovdiv; Regulations of MU-Plovdiv dated 28.01.2021 and includes the following documents:

1. Application (according to the template from the website of MU-Plovdiv) to the Rector of MU-Plovdiv for admission to official defense with a list of documents - with an incoming number and date from the Registry Office.
2. Dissertation - in 1 copy on paper and in electronic format (in PDF format) for publication in Open Science Cloud.
3. Summary* in Bulgarian and in one of the languages traditionally used in the relevant scientific field, on paper and in electronic form (in PDF format). The abstract in a foreign language is required only in electronic format (in PDF format).
4. Creative CV – European format, signed by the candidate.
5. Diploma for educational and qualification degree "master" with an appendix (notarized copies).

6. List of publications and scientific works on the subject of the dissertation.
7. Copies of the publications and scientific works on the topic of the dissertation (with the PhD student's signature).
8. Order for enrollment in PhD studies and protocol from the extended faculty council for enrollment.
9. Protocol of an extended Department council for deduction with the right of defense.
10. Deduction Order with Right of Defense.
11. Exam report - doctoral minimum in the specialty (according to the sample from the website of MU-Plovdiv).
12. Declaration of originality and authenticity of the attached documents (according to the model from the website of MU-Plovdiv).
13. Certificate of received credits from the training under the group curriculum.
14. Other documents related to the course of the procedure.
15. NACID information card for protected dissertation work in Bulgarian.
16. NACID information card for protected dissertation work in English.
17. CD/flash memory with pdf-format of the abstract (in Bulgarian and a foreign language) and dissertation, as well as doc-format of the information cards of NACID - 3 copies.

The PhD student has submitted **3 publications and one registered utility model**.

I have no remarks or comments on the documents.

2. Brief biographical data for the doctoral student

Assist. Prof. Nikolay Stantchev Zahariev was born on August 12, 1993 in Zlatograd. In 2017, he graduated from the Faculty of Pharmacy of the Medical University - Plovdiv with excellent results in the state exams. Since 2018, he has been an Assistant professor at the Department of Pharmaceutical Sciences. He was enrolled as a full-time PhD student at the same department in 2019. During his doctoral studies, he began a specialization in Drug Technology and Biopharmaceutics and completed a course in Animal Protection and Welfare (30/09/2022 – 30/09/2022, Thracian University, Faculty of Veterinary Medicine, Stara Zagora), as he uses the knowledge acquired during these studies appropriately for scientific and educational purposes.

3. Relevance of the topic and appropriateness of the set goals and tasks

The presented project of a PhD thesis with title "Nano-sized carriers for the targeted delivery of drugs for antitumor therapy" is related to the solution of an emerging problem in the therapy of oncological diseases, namely the targeted delivery of cytostatics to the malignant cells. The main objective of the work is to develop a model drug-delivery system with the potential for targeted delivery

and controlled release of the anthracycline antibiotic daunorubicin from the carrier. The assigned tasks fully correspond to the set goal and appropriately draw the experimental plan of the dissertation.

Conventional chemotherapeutics, such as the anthracycline antibiotic daunorubicin, are characterized by non-specific cytostatic action and a number of severe side effects. Specifically, for daunorubicin, these are cardiotoxicity, myelosuppression, anemia, and thrombocytopenia. Nanotechnology is one of the modern approaches that aims to increase the specificity and efficacy of antineoplastic drugs and simultaneously reduce their toxic effects. Considering these facts, daunorubicin is a suitable and properly selected drug for encapsulation in a nano-drug delivery system. The PhD student is rightly directed to use biopolymer nanoparticles as carriers for daunorubicin, as they show a better toxicological profile than synthetic ones, as well as a high degree of biocompatibility and biodegradability when ingested.

In the last few decades, polymers that change their physicochemical characteristics depending on certain parameters of the microenvironment have been widely used for targeted drug delivery. Particularly relevant for targeted therapy are those in which the release of the drug molecules included in them is determined solely by the pH variations in the body and therefore can take place at the site of action. As is known, malignant cells are characterized by a higher pH compared to healthy ones. The casein nanocarrier chosen in the thesis is particularly suitable for the purpose as it is generally recognized as safe (GRAS) and offers biocompatibility, biodegradability and bioresorbability when administered orally. Depending on the pH of the medium, the casein molecules adopt different conformations that are most energetically favorable. In an acidic environment, they thicken, and when the acidity decreases, they swell and allow the release of the loaded drugs. Another advantage of these is their ability to penetrate intracellularly through the plasma membrane in an energy-independent manner, which increases cellular uptake upon oral administration. A very good idea implemented in the dissertation at the next stage is the functionalization of the casein nanoparticles with the biopolymer of marine origin fucoidan, which is intended to improve the distribution of the drug in the body and reduce the risk of adverse drug reactions.

To evaluate the biological activity of the composite nanosystems, a series of standard *in vitro* and *ex vivo* tests were conducted, with which preclinical pharmacological studies necessarily begin. These tests characterize the cytotoxicity, extent of penetration into treated cells, and smooth muscle effects of the daunorubicin nanocomposite systems and lay a very good foundation for further pharmacological studies.

The set goal and its adjacent tasks for the development of the nanocomposite systems of the biodegradable polymer casein, functionalized with fucoidan for the targeted delivery of daunorubicin are expedient and up-to-date given the information published so far in the scientific literature. The sequence of the selected modern physico-chemical and pharmacological tests gives the presented scientific work completeness.

4. Analysis of the problem

In the State-of-the-art section of the presented thesis, in great detail are discussed the advantages of the natural polymers from the groups of proteins and polysaccharides as biotolerable, biodegradable and non-toxic carriers, and in particular casein and the polysaccharide fucoidan, used to functionalize casein nanoparticles by obtaining nanocomposite structures with optimal physico-chemical properties. Special attention is paid to both the advantages of casein as a carrier in drug-delivery systems, as well as its disadvantages and, accordingly, the possibility of overcoming

them by binding in casein-polyelectrolyte complexes. The bibliography includes 232 literary sources. Of these, 191 were cited in the State-of-the-art section, which testifies to the enormous work put in by the PhD student in reviewing the available scientific literature on the issue. The PhD student has analyzed the published information and has made a critical summary of the achievements in the field so far, described the pressing problems and scientifically justified the purpose of the dissertation work. It can be seen from the writing that the doctoral student has learned to handle scientific literature and to formulate scientific hypotheses based on the processed information.

5. Research methodology

The PhD thesis is distinguished by a rich methodology from the preparation and characterization of the casein-fucoidan composite nanostructures to the testing of their pharmacological activity. The consistent and well-argued interrelationship between the physical-chemical and pharmacological tests performed makes a good impression. The chosen methods are modern and logically find their place in the successive experimental cascade of scientific development, which contributes to justifying the practical applicability of the newly created composite nanoparticles as drug nanocarriers and reveals their potential for moving to higher technology readiness levels of development.

Casein particles were obtained by the coacervation method with subsequent spray drying, while Casein/fucoidan composite nanoparticles were obtained by polyelectrolyte complexation, cross-linking of the complex with glutaraldehyde and subsequent spray drying. The PhD student performed a significant number of experiments to establish the optimal conditions for producing both types of particles, varying the polymer/drug ratio, crosslinker concentration, and sputtering intensity. He has consistently determined the size, size distribution and zeta potential of the resulting particles by dynamic light scattering. He described their surface morphology with scanning electron microscopy and used a large set of modern methods to characterize the phase state of the medicinal substance (powder X-ray diffractometry, thermogravimetric and differential thermal analysis, differential scanning calorimetry). All these tests are correctly selected and fully characterize the physico-chemical nanocarriers, which is necessary for the subsequent pharmacological tests.

The biopharmaceutical behavior of the developed systems, intracellular penetration and distribution, as well as their *in vitro* cytotoxicity with and without loaded daunorubicin, was investigated by the PhD student on the lymphoblastic cell line Reh. The set of current tests is sufficient to reveal the advantages of the developed nanocarriers with respect to the parameters listed in the previous sentence and to determine the most suitable nano delivery system for encapsulation of daunorubicin. The PhD student has mastered the necessary methods for cell cultivation and monitoring of the biochemical characteristics of cell systems, such as activity of mitochondrial dehydrogenases and mitochondrial metabolism after exposure to the nano delivery systems. Of interest is the conducted test for interactions of nanoparticles with human serum albumin, which can affect their distribution, transport and release in *in vivo* conditions, as well as the model for studying changes in the spontaneous contractile activity of smooth muscle preparations from guinea pigs.

The statistical analysis was performed with several modern programs and shows that the PhD student has acquired a thorough knowledge of the computational methods applied for quantitative evaluation of biopharmaceutical and pharmacological data.

6. Characterization and evaluation of the PhD thesis

The presented PhD thesis is written in a high scientific style on 156 pages and is formatted according to the requirements for PhD thesis of the Medical University - Plovdiv. The texts are richly and successfully illustrated with 50 figures and 20 tables. Of these, 37 figures and 17 tables present the obtained own results. The bibliography includes 232 literary sources. A detailed analysis of the literature is made in the PhD thesis. Eight tasks have been formulated in connection with the implementation of the set goal. The materials and methods used are described with detailed protocols. The results and their discussion constitute the main part of the thesis. The results are presented accurately and completely. Eight conclusions were drawn. The conclusions are logical and cover the entire diversity of the obtained experimental data. Five contributions are listed. The bibliography includes 157 scientific sources, all in Latin. The fact that most of them are from the last 5 years proves the actuality of the research and makes a very good impression.

Nikolay Zahariev's thesis follows the common structuring scheme established in the country, respecting the ratio between the individual parts:

- 1) Introduction – 1 page.
- 2) State of the art - 41 pages.
- 3) Goals and objectives - 2 pages.
- 4) Methods and materials – 19 pages.
- 5) Results and discussion – 64 pages.
- 6) Conclusions – 1 page.
- 7) Contributions – 1 page.
- 8) References - 20 pages.

7. Contributions and significance of the development for science and practice

The contributions in the PhD thesis of Nikolay Zahariev are of a scientific and scientific-applied nature. They summarize the original experimental data obtained by the PhD student regarding a current scientific problem – targeted delivery of cytostatics to the site of action. The PhD student first proposed a new model of casein nanoparticles encapsulating the anthracycline antibiotic daunorubicin and developed a drug-delivery system with casein and fucoidan in one nanocomposite structure. Regarding the biological effects, for the first time in the present thesis smooth muscle relaxation was found after administration of casein in micromolar concentrations. The PhD student for the first time investigated the effect of daunorubicin on the spontaneous contractile activity of smooth muscle preparations from the corpus part of the stomach of a guinea pig.

In addition, the PhD thesis proposes a model for the study of spontaneous contractile activity of smooth muscles, which could be an alternative to in vitro studies of release rate, allowing assessment of the biological effect in real time. The practical focus of the contributions is very valuable for

medical practice. The registered utility model opens up very good prospects for the development and implementation in practice of a upper technology readiness level.

8. Assessment of dissertation publications

There are 3 scientific publications related to the PhD thesis. One registered utility model is also noted. An outstanding achievement is the fact that all three publications were published in prestigious international refereed journals with Impact factors varying from 5.0 to 5.4, and ranked in the highest quartile Q1. The PhD student is the first author of both the three publications and the registered utility model, and his contribution is the largest. The publications include a substantial part of the results of the dissertation work. They have been cited a total of 14 times (without self-citations) in the international database Scopus, which is an impressive achievement considering that the first publication is from the year of 2021. The large number of citations testifies to the interest of the scientific community in the published results and is evidence of the high quality and relevance of the studies. The doctoral student has participated in 6 scientific forums, of which 2 are international, 1 with international participation and 3 national. The PhD student is the lead researcher of an inter-university project at the MU-Plovdiv on the topic: "Biopolymer nano systems for targeted delivery of drugs for anti-tumor therapy" and participates in two more - one national, funded by the "Bulgarian National Science Fund" and one international under the "Program for strategic research and innovation for the development of MU-Plovdiv".

9. Personal participation of the PhD student

The PhD student Nikolay Zahariev personally participated in the conducted scientific research, as evidenced by the fact that he is the first author of the three publications and the registered utility model. The contributions described in the dissertation are a personal work of the PhD student and were obtained with the support and assistance of his scientific supervisor Assoc. Prof. Bisera Pili-cheva, Ph.D.

10. Summary of the dissertation

The Summary of the dissertation fully meets the requirements of the regulations of the Medical University - Plovdiv. The achieved results are reflected briefly and clearly, without gaps. It is written on 74 pages, containing 41 figures and 16 tables. From the summary made, a clear idea of the objectives, tasks and implementation of the dissertation work can be gained. A discussion of the results, conclusions and contributions are included, as well as a bibliography of 54 literature sources.

11. Critical remarks and recommendations

The PhD student complied with all the remarks I made during the approval of the PhD thesis; therefore, I have no critical remarks and recommendations to the conducted research and the attached set of materials.

12. Personal impressions

I know the PhD student Nikolay Zahariev from the approval of his dissertation work, held in April 2024. During his presentation, I was strongly impressed by his confidence and competence in presenting the conducted scientific research. He presented the obtained results and their significance briefly, clearly and comprehensively, in a logical sequence, which is characteristic of young scientists with a strong dedication to the scientific work and significant participation in the conducted experiments. The PhD student showed commitment to the conducted research, answered comprehensively and competently all the questions asked, and left an extremely pleasant impression of a motivated young scientist who is ready to head for the vast horizon of science with dedication and enthusiasm.

13. Recommendations for future use of dissertation contributions and results

It is more than obvious that the obtained results represent a solid basis for future research at a higher technology readiness level to obtain a pharmaceutical product with a medical application. However, the most important thing is that the research significantly enriches the available data in the world scientific literature on the use of casein molecules in combination with polysaccharide polymers for nanocomposite systems aimed at encapsulation of classic cytostatics for increasing their bioavailability and reducing their toxic effects. The obtained results are very encouraging and promising for future research in this direction. Given their high quality, I recommend continuing research with testing other polysaccharide biopolymers as well. It would also be interesting to deepen the pharmacological tests on a larger set of in vitro cell systems, in order to expand the range of target neoplasias. This would help to move to a suitable in vivo model of neoplasia, respecting the requirements of the concept of the three Rs - "reduction, refinement and replacement" of animal experiments. I would be happy if, in the future, Nikolay Zahariev continues his scientific career with the same unceasing enthusiasm and purposefulness, because in my opinion, he is already a well-established young scientist and academic teacher with excellent abilities and talent.

CONCLUSION

The dissertation of Assist. Prof. MPharm Nikolay Zaharieva *contains a number of basic and applied research results, which represent an original contribution in the field of biopharmaceutics and technology of pharmaceutical forms.* The provided scientific research **covers all the requirements** of the Law on the Development of the Academic Staff in Republic of Bulgaria (LDASRB), the Regulations for Implementation of the LDASRB and the relevant Regulations of the Medical University - Plovdiv (MU – Plovdiv) for dissertation work. The presented materials and dissertation results **fully correspond** to the specific requirements of MU - Plovdiv. I believe that the dissertation work and the presence of three publications in prestigious international scientific journals with an impact factor of 5 to 5.4, cited 14 times, as well as the registered useful model, repeatedly exceed the qualitative and quantitative criteria for acquiring the educational and scientific degree "Doctor".

The dissertation reveals that the PhD-student Nikolay Zahariev **possesses in-depth theoretical knowledge and professional skills** in the scientific specialty "Technology of dosage forms and biopharmacy" by **demonstrating qualities and skills** for independent conduct of scientific research. The high scientific value of the obtained results shows the author as an established and devoted researcher in the field of modern pharmaceutical science and the development of new pharmaceutical forms for targeted drug administration.

Due to the above, I confidently give my positive assessment of the conducted research, presented in the evaluated dissertation work, summary, achieved results and contributions, and ***I propose to the honorable scientific jury to award the educational and scientific degree "Doctor"*** to Assist. Prof. MPharm Nikolay Stanchev Zaharieva in the doctoral program in "Technology of dosage forms and biopharmacy".

Reviewer: ... Заличено на основание
Чл.5 §1, 6."В" Регламент (ЕС)2016/679

(Assoc. Prof. Maya Zaharieva, Ph.D.)