REVIEW

on a PhD THESIS entitled:

„Biological Activity of Chitosan-Based Nanomaterials"

for awarding the educational and scientific degree "DOCTOR", Higher education area 4. "Natural sciences, mathematics and informatics" 4.3. Professional field "Biological sciences"

Author: Dayana Slavchova Benkova
PhD student – independent study
PhD programme "Biophysics", BAS, Institute of Biophysics and Biomedical Engineering (IBPhBME), Department "Lipid-protein interactions"

Research supervisors:
Prof. Galya Staneva, PhD
Assoc. prof. Aneliya Kostadinova
IBPhBME-BAS

Reviewer: Prof. Dr Svetla Petrova
Department of Biochemistry, Faculty of Biology, Sofia University "St. Kliment Ohridski"
Member of the Scientific Jury appointed by order № 452/27.04.2026г. of the Director of the IBPhBME - BAS, Prof. Dr. T. Pencheva

1. General characteristics of the dissertation and the presented materials

The PhD thesis of Dayana Benkova contains 149 pages with 59 figures and 3 tables (excellently formatted), as well as 182 cited literature sources. Dissertation is written following a standard principle: Introduction and Literature Review (46 pages); Aims and objectives (2 pages); Materials and methods (22 pages); Results (31 pages); Discussion (26 pages); Conclusion (3 pages); Appendices: Contributions, list of publications (3 publications with impact factor), participation in scientific forums (3 international and 3 national); The abstract correctly presents the results and content of the dissertation. All presented materials fully meet the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB).

2. Brief biographical data

PhD student Dayana Benkova completes her Bachelor's degree in Molecular Biology at Sofia University "St. Kliment Ohridski" in 2020 and immediately thereafter obtained a Master's degree in Biochemistry at Faculty of Biology, Sofia University with an excellent defense of her MSc thesis entitled: "*Interaction of chitosan-based nanoparticles with biomimetic membranes*", prepared at the IBPhBME-BAS. Doctoral program in biophysics at IBPhBME became a natural step of her further development as a scientist. As an independent PhD student, Dayana Benkova holds various positions at IBPhBME - technician, biologist and assistant, which helped her further education and participation in all scientific projects of the Department.

3. Originality and significance of the scientific problem

The topic of the dissertation, dedicated to the *biological activity of chitosan* (CS)-based nanomaterials, encompasses the relevance of the scientific problem – nanoparticles, nanomaterials, nanotechnologies used as an innovative scientific approach nowdays. Despite the fact that nanomaterials are naturally occurring, the study of synthetically created and specifically manipulated nanomaterials is innovative and will determine the future of all human activities - medicine, electronics, energy, food industry, cosmetics, agriculture, etc. In this sense, the study of the properties of *newly created CS-based nanomaterials, the molecular mechanisms of their interaction with model and natural (fungal) membranes, as well as their potential for biological application as effective, environmentally compatible antifungal agents, is relevant and significant, because it forms the scientific foundation for future applications of CS-based hybrid nanocomposites (HNCs).*

4. General characteristics of PhD thesis

Dayana Benkova's dissertation is competently written, demonstrating excellent knowledge and analysis of scientific research in the field. The assessment of this extensive study allows her to define the working hypothesis and scientific aim of the study. The literature review presents in detail the *essence* ("natural, accidental or purposefully produced material in the range of 1-100 nm, with specific physicochemical properties, determining a variety of activities and potential applications") and *classification* of a huge variety of inorganic and organic, natural and anthropogenic nanomaterials (NMs), describing their structure, physicochemical characteristics, properties, reaction mechanisms and applications. The dissertation is dedicated to chitosan-based NMs (NCs) and therefore the structure-function relationship of chitosan is analysed in detail through its *specific physicochemical properties* (critical combination of degree of deacetylation (DD) and Mr) and *bioactivities* (intra- and intermolecular hydrogen bonds, chelating interactions with metal ions, electrostatic interactions and potential for covalent modifications).

The *structure of chitosan* defines its adhesion, aggregation, adsorption properties as well as its excellent *biocompatibility*, which make it a valuable polymer with potential diverse applications, especially biomedical (nutritional supplements, regenerative medicine, antimicrobial agents, alternative to conventional viral-vector systems in gene therapies, vaccines, drug delivery systems, antitumor agents, etc.). *Hybrid composite nanomaterials* (HNCs), combining the characteristics and nanostructural organization of NCs and HNMs, are discussed also in detail. Dayana Benkova considers *polymer-based HNCs, composed of an organic polymer and inorganic metal NPs to be the most promising and* focuses on the potential applications of hybrid CS-based HNCs with metal oxides, considering their use as effective nanofungicides against phytopathogenic fungi as *an innovative strategy*. In great detail, Dayana Benkova analyses the *composition, structure and dynamics of biological membranes*, the main physicochemical characteristics of the lipid bilayer and its phase states, the mechanisms of oxidative processes in the membranes, as well as the *biological characteristics of the studied species of phytopathogenic fungi Fusarium solani and Alternaria solani*.

Dayana Benkova formulated two aims that explore the scientific problem integrally - *mechanistically and applied*: 1. Study of the *mechanisms of interaction* between CS-based and PEG-based HNCs nanomaterials with membrane models that mimic the lipid phase heterogeneity of eukaryotic membranes and 2. *Antifungal activity* of CS-based HNCs nanomaterials containing ZnO, CuO, SiO_2, which defines the topic of the dissertation work. The specified tasks fully correspond to the aims.

The section "*Materials and Methods*" is written in an extremely detailed and informative manner, summarizing a huge amount of data from different scientific fields, which proves the *interdisciplinary nature of the scientific research*: *chemistry* (chemical synthesis of NMs); *biophysics* (dispersion and analysis of the obtained vesicles, Laurdan fluorescence spectroscopy, optical microscopy, dynamic light scattering, electrophoretic light scattering for measuring ζ-potential, diffusion); *biochemistry* (assessment of oxidative stress by determining the activity of superoxide dismutase, the level of carbonylated membrane proteins, the levels of lipid peroxidation); *microbiology* (cultivation of phytopathogenic fungi); and *statistics*. Undoubtedly, Dayana Benkova is already a well-established researcher who can conduct a complex, complete scientific study in the field of nanomaterials.

The section "*Results*" determines the main scientific and educational value of the dissertation. The results are presented logically in the sequence of the tasks set, excellently formatted and scientifically described. The main results are presented in 3 sections: *characterization of the studied NMs* for model membranes and phytopathogenic fungi; *study of the mechanisms of interactions* of the studied NMs with model membranes; and *study of the biological activity* of CS-based HNCs against phytopathogenic fungi - *A. solani and F. solani*.

The first part examines the colloidal characteristics of dispersions of NMs created for the studies with model membranes (in ddH_2O) and natural membranes of phytopathogenic fungi (in 0.2% DMSO). Although the topic of Dayana Benkova's dissertation is proving the biological activity of CS NMs, the foundation on which the scientific hypothesis should be built are the studies on their structure, physicochemical and molecular biological properties, biophysical behavior and interactions with membranes. The scientific investigation uses an innovative approach to chemically combine organic

(natural and biocompatible) CS with inorganic oxides (ZnO, CuO and SiO2) to create stable nanocomposites with different properties and activities, so the very creation of CS-based *nanocomposites is a significant contribution to the dissertation work.* A comprehensive biophysicochemical analysis of CS-based and PEG-based hybrid nanocomposites was performed and critical parameters were identified - size (DLS (hydrodynamic size) and HRTEM (actual geometric size), surface charge (ζ -potential) and morphology. The results follow a comparative analysis between *pure CS NPs* (spherical shape, 20 to 40 nm size and the highest value of the ζ - potential of +33.5 mV), the *hybrid nanocomposites* (increased average hydrodynamic size and decreased surface charge, CS-CuO - 334.3 nm и +16.1 mV, CS-SiO2 - 770 nm и 17.7 mV, CS-ZnO - 438 nm и 21.6 mV) and *PEG-based HNCs* (largest size and lowest potential, PEG-ZnO HNCs - 1116 nm и +9.48 mV). HRTEM visualized morphology of nanocomposites dispersed in water proves aggregation in CS-ZnO and PEG-ZnO HNCs, which gives a structural organization of a spherical ZnO core surrounded by a polymer layer of CS and PEG. In CS-based nanocomposites dispersed in DMSO, Dayane Benkova established differences in structure, size, potential and structure - aggregated CS core coated with a ZnO layer (CS-ZnO-HNCs).

The *second part* focuses on studying the *interactions of nanomaterials with model membranes*, using various model systems in different phase states, monitored with Laurdan fluorescence spectroscopy. The membrane models that are investigated for changes in lipid ordering are of varying degrees of complexity:

1) ***Ld** phase model* - EggPC LUVs. EggPC LUVs, treated with 0.01 mg/ml pure CS NPs. show enhanced lipid order compared to control EggPC LUVs without CS. GP dependence of CS NPs concentration is hyperbolic with increasing NMs concentration (saturation effect), and the calculated quantitative changes in lipid ordering in the Ld phase induced by NMs show the order: CS > CS-CuO > CS-ZnO > CS-SiO_2 > PEG-ZnO.

Dayana Benkova concludes that the tested NMs increase the ordering of all lipid phases, reducing the polarity in the region of the lipid glycerol backbone, and this effect is dependent on the initial lipid ordering and polarity of the bilayer (confirms the established "ordering" effect of CS NMs, due to: *electrostatic interactions* (cationic charge of CS NPs (+34 mV) and negatively charged EggPC membranes (-19 mV); *hydrogen bonds* with choline polar head of EggPC and *adhesion* of CS NPs to the membrane surface; *increase in the viscosity of the microenvironment* in the region of glycerol; free diffusion of CS NPs (40 nm) in the bilayer and hydrophobic interactions). Dayana Benkova also analyzed the influence of solvents (acetic acid, DMSO) used in the NMs experiments to determine the actual change in GP. The interaction of CS NPs and CS-CuO NMs with EggPC LUVs was monitored by DLS and ELS. The **ζ - potential** of decorated vesicles obtained after treating of EggPC LUVs with CS NPs (+33.5 mV), sharply shifts from –19 mV to +19 mV at pH 5.5 that affects the adhesion on the membrane surface. Dayana Benkova discusses DLS results in great detail and make a conclusion that the studied NMs – CS-NPs and CS-CuO HNCs – have *full interaction and matterial exchange with* EggPC LUVs *vesicles.*

2) ***Lo** phase model* of raft-like domains in the eukaryotic cell membrane - 2 types of binary mixtures - EggPC/Chol (1:1) and EggSM/Chol (1:1). The main conclusion from these studies is that the *"ordering" effect on the EggPC/Chol Lo phase is 3x greater compared to the effect on the raft-like EggSM/Cho Lo phase*. The presence of cholesterol in the membranes The presence of cholesterol in membranes is responsible for the reduction of surface charge, electrostatic interactions and weaker adhesion of NMs to the membrane surface. Therefore, the measured quantitative changes in lipid ordering in raft-like Lo phase of EggSM/Chol are 2x smaller than those in Ld phase. In contrast, with increasing *CS-SiO_2* concentration, GP values exponentially decrease - an indicator of the "fluidizing" effect. The quantitative changes in lipid ordering of the raft-like Lo phase induced by NMs are distributed as: CS-CuO ≈ CS-ZnO > PEG-ZnO > CS > CS-SiO_2. The relationship is analogous for the binary mixture of EggPC/Chol (1:1), which also forms a lipid pattern in the Lo phase, although more disordered than that of EggSM/Chol. The quantitative changes follow the row: CS-ZnO > CS-CuO > CS ≈ PEG-ZnO > CS-SiO_2.

3) *model of the eukaryotic plasma membrane (Ld/Lo coexistence*, with different degrees of ordering and phase heterogeneity) - a ternary mixture of EggPC/EggSM/Chol in the ratio 2:2:1 (phase separation in the bilayer at physiol. T °C, EggSM and Chol are organized in the Lo phase in the form of

domains surrounded by a continuous Ld phase of EggPC). In this model, *all tested NMs cause an ordering effect* on the heterogeneous model, with pure CS NPs showing a concentration-dependent increase in GP values, while the effect of CS-based HNCs is much weaker. PEG-ZnO HNCs show a stronger effect than other hybrid NMs. The quantitative changes in lipid ordering in EggPC/EggSM/Chol LUVs (Ld/Lo coexistence) are expressed in the following row: CS > PEG ZnO > CS-ZnO > CS-CuO > CS-SiO_2. Dayana Benkova shows a certain degree of preferentiality, as cationic CS nanoparticles interact predominantly with the liquid Ld phase of heterogeneous membranes compared to the Lo phases (high cholesterol content, more packed, reduced negative surface charge).

Morphology changes of GUVs induced by different concentrations of NMs were analyzed through properties as adhesion, fusion, contraction, pore formation, tubule formation and membrane permeabilization in model membrane systems in different phase states – Ld (EggPC GUVs,) and heterogeneous Ld/Lo (EggPC/EggSM/Chol GUVs). In this large-scale study, Dayana Benkova made a *conclusion that integral changes were observed to varying degree - increased lipid ordering, changes in the permeability of the bilayer, membrane invaginations and membrane disintegration, different times of collapse and adhesion*. For EggPC GUVs treated with *CS-ZnO HNCs*, a 2x higher concentration is required to achieve a similar degree of morphological changes as when treated with CS NPs. (inducing the greatest changes in lipid arrangement at lowest concentration threshold of 40 μg/ml).

In order to assess the *biological significance of lipid-NMs interactions* in heterogeneous eukaryotic cell membranes, it is very important to analyze in depth the morphology of heterogeneous EggPC/EggSM/Chol GUVs (Ld/Lo phase coexistence) treated with increasing concentrations of CS-based NPs. Fluorescence microscopy with unsaturated Rhodamine-PE (definitive for the Ld phase) showed *the existence of two types of morphological transformations – membrane invaginations and liposome shrinkage*, more pronounced for Ld and less for the Lo phase at high concentrations. A critical concentration of 160 μg/ml of CS NPs was determined, at which irregular distribution and interactions of NPs across the entire membrane surface trigger membrane invaginations and membrane disintegration.

The ***biological activity*** of CS-based HNCs against phytopathogenic fungi - *A. solani and F. solani*, as well as the potential applications of the newly created nanomaterials are analyzed in the *third part* of the dissertation. Applying a combination of microbiological, biochemical and biophysical approaches, Dayana Benkova presents antifungal and prooxidant (exogenous induction of ROS) activities. The combination of the physicochemical characteristics of the nanocomposites (size, shape, surface charge) and reactivity/nanotoxicity (redox-surface catalysis of inorganic oxides) was used to optimize the synergistic antifungal efficacy, highly dependent also on their diffusion potential for internalization in fungal membranes.

The first set of experiments is an evidence for existence of *biological antifungal activity*. Here, a model system using a fungal spore suspension treated with HNCs at different concentrations and a classical method for determining the size of the inhibition zones formed around the wells with fungal culture was applied. As a prerequisite for highly efficient interaction with fungal cell membranes, the synthesized HNCs were further processed so that the amino groups of CS were maximally protonated and efficiently dispersed in DMSO (smaller colloidal sizes and greater positive charge). The results show *a strong antifungal effect of all tested HNCs, most pronounced when treated with CS-ZnO HNCs and a higher sensitivity of A. solani compared to that of F. solani.*

Dayana Benkova proposes a probable molecular mechanism of biological action of CS-based HNCs on phytopathogenic fungi - *induction of ROS and OS*, which is proven by analyzing the markers of oxidative stress and possible changes in the lipid arrangement of fungal membranes. The results clearly show *increased fungal SOD activity* as a result of the action of all tested CS-based HNCs on *F. solani* (submerged culture), with *the most significant SOD activity* (21 U/mg protein, 2.5x higher than that of the control variant) being demonstrated upon treatment with 0.5 mg/ml *CS-CuO HNCs*. Increased SOD activity in *A. solani* was found only upon treatment with CS-SiO_2 HNCs. According to Dayana Benkova, the lower levels of SOD or its absence upon treatment with the other HNCs in *A. solani* cells is an indication of rapid dismutation of $O2^{\bullet-}$ by SOD.

Protein carbonylation assay (PCC, marker for existence of irreversible post-translational oxidations of certain aminoacids's side groups of by $OH^{\bullet}$), showed that all tested CS-based HNCs caused *increased PCC levels in fungal cells compared to controls*. The increase in PCC was *dose-*

dependent and had the highest effect when treated with 1 mg/ml CS-CuO HNCs in both phytopathogen species (*F. solani* with higher sensitivity). The assay of malondialdehyde (MDA) - a marker for lipid peroxidation, proves the already established pattern - *all CS-based HNCs increase MDA levels in fungal cells in a dose-dependent manner*, with the greatest effect again being found in treatment with CS-CuO HNCs of *F. solani* (4x higher MDA levels), while *A. solani* responds to the action of CS-CuO HNCs to a 2x lower extent (MDA above 100 nmol/ml). The greatest effect in *A. solani* is found in treatment with *CS-SiO_2 HNCs* (1 mg/ml), where 2.6x higher MDA levels (180 nmol/ml) are determined. Dayana Benkova suggests that the *strong prooxidant activity of CuO is due to redox-surface catalysis and the release of Cu^+ and Cu^{2+}, participating in Fenton and Haber–Weiss- like non-enzymatic reactions* in cells, while *SiO_2 acts through its nanostructural organization* in the composition of HNCs (increasing the number of highly reactive structural defects on the particle surface), which changes the redox potential. *CS-ZnO HNCs do not exert a significant antifungal effect.*

The changes in the lipid ordering of the fungal membranes were analyzed by Laurdan fluorescence spectroscopy. The most important result here (more pronounced on the membranes of *F. solani*) are the *increased GP values in both strains after treatment with CS-CuO HNCs*, evidence of the increased lipid ordering in the membranes. In agreement with the obtained results of oxidative stress, the hybrid nanocomposite of *CS-SiO_2 induces a dose-dependent increase in membrane ordering only in A. solani. CS-ZnO HNCs cause opposite effects on the two fungal species* - an increase in membrane ordering in *A. solani*, but a decrease in membrane ordering (fluidizing effect) in *F. solani*. Dayana Benkova proposes a mechanism for the increased lipid ordering in model membranes, which, however, must take into account the presence of protein components in natural fungal membranes and include some additional mechanisms. Deyana Benkova believes that there is a certain correlation between the models and real membranes, which demonstrates the key role of the lipid component in the membrane in contact with exogenous molecules such as NMs.

Dayana Benkova summarizes her research in a special section "*Discussion*", where she presents in detail her hypothesis about the mechanisms of interaction of the studied nanocomposites with fungal membranes, considering the ability of positively charged antifungal agents to pass through the cell wall and fungal plasma membrane as a key factor in disrupting the integrity of the cell wall and determining their effectiveness, *i.e. a combined mechanism involving the induction of oxidative stress and significant changes in the lipid organization of fungal membranes.*

I consider the discussion part of the dissertation as a very important section for evaluating, comparing and integrating all experimental options in order to *present biological meaning and significance* of the entire study. The main contribution of Dayana Benkova's dissertation is formulated as "*the scientific basis for future application of CS-based hybrid nanocomposites as effective and environmentally friendly antifungal materials for the control of phytopathogenic fungi Alternaria solani and Fusarium solani in agriculture, with potential for use in plant protection and development of sustainable nanopesticides against resistant strains*".

5. Publications and participation in scientific forums

Dayana Benkova presents the obtained results in 3 publications in specialized journals (2 in *International Journal of Biological Macromolecules* IF 7.7, Q1), *Proceedings of the Bulgarian Academy of Sciences*, IF 0.3, Q3) with a total impact factor of 15.7, for which more than 40 citations have been noted, as well as participation in 6 conferences (3 with international participation and 3 national), at which she was awarded 2 awards - *Diploma for an excellently presented report by a young scientist* (2024) and the "*Ivan Evstratiev Geshov" Award for the youngest scientists of the Bulgarian Academy of Sciences* (2025). In two of these publications, the PhD student is a second author and first in one, which is a proof of her personal contribution. Dayana Benkova's PhD thesis has been funded and partially included in 6 ongoing and 4 completed scientific projects. The submitted dissertation fully meets the minimum national requirements (under Art. 2b, para. 2 and 3 of LDASRB) and the additional requirements of BAS for the acquisition of the educational and scientific degree "doctor".

6. Fulfillment of the minimum state requirements and the individual plan

Doctoral student Dayana Benkova has successfully completed her individual doctoral plan and has taken all the exams provided for in it. The certificate of compliance with the minimum requirements for

the ONS "Doctor" for professional field 4.3. Biological Sciences has been filled in correctly. Of the required 80 points, Dayana Benkova has acquired 115 points. This includes: a successfully approved dissertation work - 50 points, 2 articles in a journal with Q1 - 50 points, 1 article with rank Q3 -15 points.

7. Questions

Since the topic of the dissertation concerns the biological activity of nanomaterials, I have some biological questions:

1. What is the structure of fungal SOD enzymes and how the studied HNCs would affect their catalytic mechanism and activity?

2. According to the PhD student, "the lower levels of SOD upon treatment with CS-SiO_2 and the lack of enzymatic activity upon treatment with the other HNCs in *Alternaria solani* cells is an indication of rapid dismutation of O2•− by SOD". Explain this conclusion.

3. Since the fungal SOD enzymes are activated and perform a protective function upon fungal infection of host plants, the treatment with the studied HNCs should be done in a more complex biological system, including a specific plant infected with *Fusarium/Alternaria*. Have you made or intented to do experiments investigating the influence of the studied HNCs on phytopathogen-host relationships?

8. Conclusion

Dayana Benkova demonstrates excellent knowledge of the problem and the ability acquired in the course of the work to interpret the obtained results, to propose hypotheses and to compare them with already published data. Knowing Dayana Benkova from her years as a student at Sofia University "St. Kliment Ohridski", I can express my excellent impressions of her and the demonstrated purposefulness and responsibility.

Having become acquainted with PhD thesis and the accompanying scientific papers presented in the procedure and based on the analysis of their significance, scientific and applied contributions, I confirm that the presented dissertation and the scientific publications to it, the quality and originality of the results and achievements fully meet the requirements of the LDASRB and the Regulations for its implementation for the acquisition of the educational and scientific degree "doctor" in the scientific area 4. Natural sciences, mathematics and informatics, professional field 4.3. Biological sciences (Biophysics).

Based on the above, I recommend to the Scientific jury to award Dayana Benkova the Educational and Scientific degree "Doctor" in the professional field 4.3. Biological Sciences (Biophysics).

26.06.2026
Sofia

Member of the Scientific Jury

Prof. Svetla Petrova, PhD
(Department of Biochemistry,
Faculty of Biology, SU)