

REVIEW

from

Prof. Biliana Pancheva Nikolova-Lefterova,

Institute of Biophysics and Biomedical Engineering, BAS

About: PhD thesis for award on educational and scientific " doctor " degree in area on higher education 4. Natural sciences, mathematics and computer science, professionally direction 4.3 Biological Sciences, doctoral program " Biophysics", section "Lipid-protein interactions" - IBFBMI-BAS.

Author: Dayna Slavchova Benkova

Topic: "Biological activity of chitosan - based nanomaterials"

Scientific supervisors:

Prof. Dr. Galya Staneva

Assoc. Prof. Dr. Anelia Kostadinova

1. Relevance and significance of the scientific problem being developed

The presented dissertation is dedicated to an extremely relevant and promising area of modern biophysics and nanobiotechnologies – the study of the biological activity of chitosan -based nanomaterials and their interactions with biological membranes and phytopathogenic microorganisms.

Over the past two decades, nanomaterials have established themselves as one of the fastest growing areas in biomedical and biotechnology. Of particular interest are biocompatible polymeric nanomaterials that can serve as drug carriers, antimicrobial agents, or functional components of intelligent biomaterials. Among them, chitosan occupies a special place due to its biocompatibility, biodegradability, low toxicity, and ability to be chemically modified.

However, the mechanisms of interaction between nanomaterials and cell membranes are still not fully understood. Understanding these interactions is essential for both the medical applications of nanomaterials and the assessment of their safety and efficacy.

Another important aspect of the dissertation work is the search for new means for the control of phytopathogenic microorganisms. In the context of increasing resistance to conventional fungicides and the need for sustainable agriculture, the development of environmentally safe antimicrobial systems is a significant scientific and societal task.

Therefore, the topic of the dissertation is undoubtedly relevant, scientifically significant and fully corresponds to modern trends in the development of biophysics, nanobiotechnologies and applied biology.

2. General characteristics of the dissertation work

The dissertation is developed on 148 pages and includes all the necessary structural components of a modern scientific study - introduction, literature review, goals and objectives, materials and methods, results and discussion, conclusion, scientific contributions, publications on the topic and literature used.

The structure of the dissertation is logical, consistent, and well-balanced. The individual sections are interconnected and lead consistently to the solution of the scientific tasks set.

The high quality of the dissertation's layout is impressive. The text is written in good scientific language, the results are presented clearly and are illustrated through many figures, tables, and diagrams.

3. Evaluation of the literature review

The literature review is in-depth and demonstrates the doctoral student's excellent awareness of the state of research in the field.

The following were considered:

- the structure and functions of biological membranes;
- the organization of lipid domains;
- the properties of chitosan;
- methods for obtaining and characterizing nanomaterials;
- the biological effects of metal oxides nanoparticles;
- the antifungal activity of nanocomposite systems.

The critical approach to the analysis of the literature data is particularly positive. The author does not limit herself to a descriptive presentation of the information but makes comparisons between different scientific concepts and outlines unsolved problems in the field.

The literature review successfully fulfills its main function - it creates the necessary theoretical basis for formulating the goals and objectives of the dissertation.

4. Evaluation of goals and objectives

The main goal of the dissertation is clearly formulated and is aimed at studying the interactions between chitosan -based nanomaterials and model membrane systems, as well as evaluating their antifungal activity.

The tasks set are specific, logically justified and fully consistent with the set goal.

After analyzing the presented results, it can be concluded that all the tasks set were successfully completed.

5. Evaluation of the methods used

The methodological approach represents one of the strongest aspects of the dissertation work.

A wide range of modern methods was used, including:

- dynamic light scattering (DLS);
- electrophoretic light scattering (ELS);
- HRTEM analysis;
- laurdan fluorescence spectroscopy;
- phase-contrast and fluorescence microscopy;
- method for forming large unilamellar vesicles;
- tests for antifungal activity;
- oxidative stress markers;
- modern statistical methods.

The selection of methods is fully adequate to the tasks set.

I particularly appreciate the combination of biophysical, physicochemical, and microbiological approaches, which allows for a comprehensive study of processes at different organizational levels.

6. Analysis and evaluation of the results obtained

The most significant contribution of the dissertation is the systematic study of the interactions between various chitosan- based nanomaterials and model membrane systems.

nanomaterials have been studied and described, depending on their subsequent application. It has been established that the combination of chitosan with different metal oxides leads to significant changes in the colloidal characteristics of the resulting hybrid nanocomposites, the data are presented in Table 2. Images showing the morphology of the nanomaterials obtained using the HRTEM method are shown in Figure 38. The results of the characterization of the nanomaterials for the treatment of phytopathogenic fungi (dispersed in 0.2% DMSO) are presented in Table 3 and Figure 39.

Table 2 also presents results from the characterization of PEG- ZnO HNCs, and then there are no results presented with these nanocomposites, why?

The next chapter discusses the interaction of nanomaterials with model membranes. Model membranes are of three types depending on their composition, with each subsequent step of the system becoming more complicated. Monocomponent model membranes represent a model of Ld the phase of cell membranes, the bicomponent EggSM / Chol LUVs serve to form a lipid pattern in the Lo phase with raft-like domain analogues in the eukaryotic cell membrane, EggPC / Chol LUVs were used as a model of the Lo phase, while the three-component EggPC / EggSM / Chol LUVs are considered the most suitable model of the eukaryotic plasma membrane of all the models in this study, due to their lipid composition and phase heterogeneity. It was found

that when homogeneous vesicles were treated with chitosan nanomaterials, the laurdan spectrum shifts to the lower wavelength, indicating higher lipid ordering.

Clearly pronounced concentration-dependent effects on lipid organization have been demonstrated in homogeneous, membrane models (Figure 41A), as well as quantitative changes in lipid organization, depending on the type of nanomaterials applied.

In the second type of model membranes EggSM / Chol, an increase in the GP value up to 40 μ g/ml is observed (Figure 42 A, B, C, D). It is interesting to note that the nanocomposite CS-SiO₂ strongly differs from the others, causing an exponential decrease in the GP value after treatment with concentrations of 40 μ g/ml.

In the Lo lipid phase model, the changes that occurred after treatment with the nanocomposites are in one direction – a concentration-dependent increase in ordering, figure 43.

In vesicles formed from the ternary mixture EggPC / EggSM / Chol, an ordering effect was also observed. It was most pronounced after treatment with pure chitosan. nanoparticles, followed by PEG- ZnO figure 44.

Particularly interesting are the results showing that different nanomaterials induce different degrees of ordering of lipid membranes depending on their phase state.

Why are only liposomes formed from a monocomponent mixture treated with 1% acetic acid (the solvent in which the nanocomposite materials are prepared)?

polydispersity and ζ -potential) of homogeneous liposomes after treatment with pure chitosan were found. nanoparticles and CS- CuO HNCs figure 45.

Of significant scientific interest are also the observed morphological transformations of the giant unilamellar vesicles, including adhesion, fusion, invagination, and membrane disintegration processes. These results provide valuable information on possible mechanisms of interaction between nanomaterials and cell membranes.

The second part of the dissertation is dedicated to the antifungal activity of chitosan -based nanocomposites against two strains of phytopathogenic fungi *A. solani* and *F. solani*.

Both strains were affected by the action of HNCs. The greatest antifungal activity was shown by CS- ZnO nanocomposites figure 54.

Oxidative stress markers were also investigated: MDA marker for lipid peroxidation and SOD as an enzyme of the cell's antioxidant defense. It was found that when treated with *F. solani* strain with CS- CuO (0.5 mg/ml) the highest levels of the enzyme superoxide dismutase were recorded (Figure 55). For the other two nanocomposites, a concentration dependence was established. While for strain *A. solani* effect was observed only after treatment with CS- SiO.

Why is there no value for superoxide dismutase after treatment with 1 mg/ml CS- CuO, is it a zero value or is it omitted?

PCC levels increased after treatment with all tested CS-HNPs, with a dose-dependent effect only observed with CS- CuO and CS- ZnO nanocomposites in both strains Figure 56. Similar results were obtained for MDA levels Figure 57.

fungus strains with CS-HNPs are examined. The chosen concentrations are again 0.5 and 1.0 mg/ml. After treatment with CS- CuO Higher GP values were reported in both strains, which is a marker for increased lipid ordering, with the effect being significantly stronger in the *F. solani* strain. Figure 58A. Interesting results were obtained after treatment with CS- ZnO, dormant strain *A. salt* has a clearly pronounced concentration-dependent fluidizing effect, while in *F. solani*. The low concentration stiffens the membrane, while the higher one slightly fluidizes it, Figure 58C.

The obtained results show promising potential for the application of CS-HNPs as an alternative to traditional fungicides.

7. Scientific and applied scientific contributions

I accept the contributions formulated by the author and believe that they can be summarized in the following main directions:

Scientific contributions:

- New knowledge has been gained about the interactions between chitosan -based nanomaterials and model membranes.
- Regularities have been established between the structure of nanomaterials and the induced changes in lipid organization.
- Mechanisms of membrane interaction have been elucidated by combining spectroscopic and microscopic methods.
- New data have been obtained on the role of the phase state of membranes in their interaction with nanoparticles.

Scientific and applied contributions:

- The potential of chitosan -based nanocomposites as antifungal agents has been proven.
- A basis has been created for the development of new environmentally safe plant protection products.
- The results obtained can be used in the development of functional nanomaterials for biomedical applications.

8. Publications

The results of the dissertation have been published and presented at scientific forums. This shows that the results obtained have received scientific recognition and have become known to the international scientific community.

I believe that publication activity is completely sufficient for acquiring the educational and scientific degree of "doctor".

9. Critical notes, questions and recommendations

I have no significant critical comments about the dissertation.

I have the following questions:

- *Table 2 also presents results from the characterization of PEG- ZnO HNCs, and then there are no results presented with these nanocomposites, why?*
- *Why are only liposomes formed from a monocomponent mixture treated with 1% acetic acid (the solvent in which the nanocomposite materials are prepared)?*
- *Why is there no value for superoxide dismutase after treatment with 1 mg/ml CS- CuO, is it a zero value or is it omitted?*

I would recommend that future research include:

- experiments on cell models;
- assessment of cytotoxicity on different cell types;
- expanding research on other groups of pathogenic microorganisms;
- study of the possibilities for practical application of the developed nanocomposites .

These recommendations do not in any way diminish the high scientific value of the dissertation.

CONCLUSION

The presented dissertation work of Diana Slavchova Benkova represents a complete, independent and original scientific study with clearly expressed scientific and scientific-applied contributions.

The doctoral student demonstrates in-depth theoretical knowledge, excellent command of modern experimental methods, the ability to critically analyze results, and skills in conducting independent research work.

The dissertation fully meets the requirements of the Law on the State of the Republic of Bulgaria on the Protection of Biological and Biological Resources, the Regulations for its implementation and the regulatory documents of the Institute of Biophysics and Biomedical Engineering at the Bulgarian Academy of Sciences, for the acquisition of the educational and scientific degree "doctor".

I confidently give a positive assessment of the dissertation work and propose to the Scientific jury to award Dayana Slavchova Benkova the educational and scientific degree "doctor" in professional field 4.3. Biological Sciences, doctoral program "Biophysics".

Date: *19.06.2026*

Reviewer: