To the Chairman of the Scientific Jury
(determined by Order No. 605/10.08.2023
of the Director of the Institute of Biophysics and
Biomedical Engineering, BAS

REVIEW

regarding a PhD work for the acquisition of the educational and scientific degree "Doctor" in professional direction 4.3 "Biological Sciences", Doctoral program "Biophysics",

Scientific consultant: Prof. Dr. Galya Staneva,
Author of the dissertation: Vesela Vasileva Yordanova,
independent study doctoral student,
Dissertation topic: "MEMBRANE REORGANISATION UNDER OXIDATIVE STRESS: EFFECT OF OXIDIZED LIPIDS",
Lipid-Protein Interactions Section,
Institute of Biophysics and Biomedical Engineering, BAS
Reviewer: Prof. Iana Christova Tsoneva, DSc,
Institute of Biophysics and Biomedical Engineering – BAS

Vesela Yordanova graduated from Sofia University "St. Kliment Ohridski", Sofia, Faculty of Biology, master's degree in Biotechnology. She was enrolled as a doctoral student of independent training on 1.08.2020. According to the procedure, Yordanova has submitted all the necessary documents in accordance with the Regulations for the application of the ZRASRB for the opening of a protection procedure.

Description of the dissertation:

The presented dissertation is written on 151 pages and 64 figures, and is structured in the standard way, consisting of the following sections: Abbreviations used, Introduction, Literature review, Aim and objectives, Materials and methods, Results and discussion, Conclusion, Contributions, Publications in dissertation, Participation in conferences,

Declaration of the originality of the results, Literature references, including 417 literary sources.

Relevance of the topic:

Recently, ω-3 unsaturated fatty acids (PUFAs) have been scientifically relevant, as they are extremely important not only for nutrition, but also for health, alleviating a number of inflammatory diseases and also autoimmune processes such as atherosclerosis, neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and multiple sclerosis, rheumatoid arthritis, diabetes and lupus. Polyunsaturated fatty acids are widely represented mainly in the plasma membrane and are mainly responsible for the fluidity of the membranes and the reorganisation of the dynamic structures of the "rafts" type, discovered and popularized only in 2006. PUFAs are highly susceptible to oxidation by reactive oxygen species in cells and the authors set out to investigate the effect of two of the most physiologically active oxidized lipids POVPC (1-palmitoyl-2-(5'-oxo-valeroyl)-*sn*-glycero -3-phosphatidylcholine) and PGPC (1-palmitoyl-2-glutaryl-*sn*-glycero-3 phosphatidylcholine) on the structural organization of the lipid bilayer of biomimetic systems (liposomes) that model the phase states of the membrane. The liposomes used are mainly phosphatidylcholine (PC) molecules with varying degrees of unsaturation of the fatty acid in the *sn-2* position. The following were used: 1) the monounsaturated lipid POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3 phosphatidylcholine), containing oleic acid, and 2) the polyunsaturated PDPC (1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3 phosphatidylcholine), which contains docosahexaenoic acid (DHA - 22:6 ω-3), which is a target of oxidative stress but also exhibits a number of pleiotropic properties.

Special attention has also been devoted to the study of secretory phospholipases ($sPLA_2$), which lead to the production of free fatty acids and lysolipids at the *sn-2* position of glycerophospholipids. The products of the action of secretory phospholipases on phospholipids with more unsaturated fatty acids are associated with oxidative stress and also some inflammatory processes.

In conclusion, a more complete knowledge of the impact of oxidized phospholipids on the membrane structure and related processes at the molecular level could provide some

diagnostic and therapeutic approaches in many widespread diseases such as neurodegenerative and atherosclerosis.

Literature review:

The literature review is appropriately systematized, balanced and represents a good scientific review of modern literary data related to the topic of doctoral studies. The presented overview is illustrated with 16 figures, current and contemporary literary sources are cited. At first glance, the literature review seems too extensive, but in fact, without considering some novelties regarding recent research in the field of biological membranes, it would be difficult to motivate an up-to-date scientific research using model biomimetic membranes. All this shows that the doctoral student has good theoretical training. In conclusion, the literature review is written in relation to the objectives of the research conducted.

Purpose and tasks:

The goals and tasks are clearly formulated, related to the study of the influence of biologically oxidized lipids on the lateral organization of unilamellar vesicles and the activity of phospholipase A_2 ($sPLA_2$) in the same biomimetic systems composed of lipids with different degrees of unsaturation at the *sn-2* position of fatty acids. acids. For this purpose, the tasks are organized into 7 specific tasks, the first of which has 2 subtasks. In general, the tasks meet the objectives: Creating different types of biomimetic systems containing oxidized lipids depending on the lamellarity and modeling different phase states and phase transitions; tracking the degree of unsaturation of the fatty acids incorporated at the *sn-2* position in the molecule of GPLs, POPC and PDPC, on the "rafts" type domains at physiological temperature by quenching the DPH fluorescence by the TEMPO molecule; Laurdan fluorescence spectroscopy to determine the differential effect of oxidized lipids; Fluorescence spectroscopy to determine the influence of the degree of unsaturation of fatty acids incorporated in the *sn-2* position in a mixture of three types of lipids on the enzymatic activity of $sPLA_2$ at physiological temperature; Determination by fluorescence spectroscopy of the activity of $sPLA_2$ as a function of the degree of unsaturation at the *sn-2* position of phosphatidylcholine in binary, ternary and

quaternary mixtures, as well as analysis of the rate of the enzyme reaction in systems that mimic the liquid-disordered (L_d) phase condition and L_o/L_d coexistence of different combinations of lipids.

Materials and methods:

High quality lipids purchased from Avanti Polar Lipids were used. Their structural formulas are also presented. Appropriate fluorescent labels and probes such as 1,6-diphenyl-1,3,5-hexatriene (DPH), (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) and 6-dodecanoyl-N ,N-dimethyl-2-naphthylamine (Laurdan) as well as phospholipase A_2 and suitable buffers. As an omission, I would point out that the unit in which the molecular weight is determined is not specified. The methods are detailed and could be used as a guide for young staff such as graduates or PhD students. Also included are 3 figures that show the mechanism of action.

Results and discussion:

When studying the influence of the oxidized lipids POVPC and PGPC and the degree of unsaturation of the fatty acid at the *sn-2* position in the PC molecule on the formation and size of the "rafts" domains by DPH-TEMPO fluorescence spectroscopy, it was proven that phosphatidylcholine liposomes - a model of L_d phase, are characterized by the lowest values of Q (the ratio of fluorescence intensities with TEMPO and without TEMPO), and the mixture SM/Chol (50:50) (model of L_o phase) is characterized by the highest. The remaining mixtures, binary and ternary, which are mfdel of L_o/L_d coexistence are located between these values (Fig. IV.1). The addition of POVPC increased the formation of the L_o phase more compared to the use of PGPC (Fig. IV.2). The addition of POVPC and PGPC leads to the formation of larger shelf-like domains (Fig. IV.3).

The temperature dependence of the general polarization (GP) of Laurdan fluorescence calculated from the obtained emission spectra in pure phosphatidylcholine liposomes shows a decrease in the GP values of this probe with increasing temperature. GP strongly depends on the presence of oxidized lipids and cholesterol, and also on the combination of lipids in the formation of liposomes (single-component, binary, or ternary) (Fig. IV.4-10).

The activity of phospholipase A_2 (bee $sPLA_2$) was also investigated by fluorescence spectroscopy as the vesicles were composed of phospholipids with different degrees of unsaturation of the fatty acids in the POPC and PDPC molecule. Included in the vesicles is a fluorogenic substrate of this phospholipase called PED6 (a phosphatidylethanolamine molecule labeled with the fluorescent dye BODIPY to the *sn-2* acyl chain). Enzyme activity was higher in monounsaturated membranes than in polyunsaturated ones, which is consistent with results obtained by other authors (Fig. IV-11). The doctoral student found that in presence of OxPC the enzyme activity of phospholipase A_2 strongly depends on the lipid hydration protocol (Fig. IV.12.1-2 and Fig. IV.12.3) in both types of matrices.

The presence of cholesterol in different concentrations affects the rate of the enzyme reaction, which is higher in monounsaturated membranes than in polyunsaturated ones (Fig. IV 13.1-3).

When studying the degree of unsaturation of fatty acids in the glycerophospholipids POPC and PDPC molecule on the activity of $sPLA_2$ in PC/SM/Chol mixtures used in different molar ratios (model of L_o/L_d model co-existence) it was shown that in polyunsaturated membranes, the lowest enzyme activity is at an equimolar lipid ratio in the PDPC/SM/Chol 1:1:1 mixture, i.e. with the highest content of SM and Chol (Fig. IV-14.1-3).

The effect of oxidized lipids (OxPCs) on $sPLA_2$ activity in monounsaturated and polyunsaturated PC/SM/Chol membranes in lipid ratio 1:1:0.5 shows that OxPCs exhibit a strong inhibitory effect on enzyme activity in both types of lipid matrices, which for POVPC is 100%, and for PGPC – nearly 90% (Fig. IV-15). The slopes of the kinetic curves show that the lowest rate of the hydrolysis reaction catalyzed by sPLA2 was found for quaternary PC/SM/Chol/OxPC 1:1:0.5:0.3 mixtures (Fig. IV-15.3B). In the OxPC-containing membranes, the enzyme reaction rate decreased by over 85% compared to the ternary control mixtures. In conclusion, the doctoral student analyzes the kinetics of the hydrolysis of phosphatidylcholine membranes in the L_o/L_d phase state under the action of $sPLA_2$, that it is extremely complex, related to the lateral phase separation, membrane heterogeneity and curvature, as a result of the accumulation of single-chain PCs

(lysoproducts). and free fatty acids that can remain in the bilayer, providing a different character to the membrane structure.

I accept the 9 conclusions and 3 contributions made by the doctoral student, which are **presented quite compactly and correspond to the results obtained.**

The specified contributions relate to the field of obtaining and proving facts new to science. The indicated conclusions could help to clarify the structural organization and physicochemical properties of biological membranes, as well as provide mechanisms of pathologies related to oxidative stress and aging, discussed in the dissertation.

The synopsis corresponds to the dissertation.

Scientific publications in the dissertation:

1. Yordanova V. at al, International Journal of Molecular Sciences, 24, 11166, 2023, Q1, IF 5.6

2. Yordanova V. at al, Comptes rendus de l'Académie bulgare des Sciences, 74, 78-87, 2021, Q2, IF 0.343

3. Yordanova V. at al, Oxidation Communications, 43, 4, 678-687, 2020, Q3, SJR 0.224

Vesela Yordanova is the first author of all publications. I know the PhD student and her work over the years and I accept that most of the results are her personal contribution.

Participation in conferences:

The participation of the doctoral student with **oral or poster reports** at our and international events is 8. The doctoral student is the first author of all of them.

I have no critical remarks or questions for the doctoral student, but I would recommend visualising the vesicles in the future.

Conclusion:

The dissertation is current. A considerable volume and quality of scientific research work has been carried out with appropriate and modern biophysical methods. The obtained new and original results have been published in 3 journals (1 - Q1, 1 - Q2 and 1 - Q3). The dissertation is a voluminous and correctly conducted scientific research, to a large extent the personal work of the doctoral student, which deserves an undeniably positive evaluation. The dissertation in terms of the relevance of the research, the quantity and quality of the conducted experiments, the number of publications and the significance of the conclusions and contributions, fully complies with the Law on the Development of the Academic Staff in the Republic of Bulgaria and the Regulations for its application at the IBFBMI-BAS. All this gives me reason to give my positive assessment and to confidently recommend to the members of the Scientific Jury to vote positively for awarding Vesela Vasileva Yordanova the educational and scientific degree "doctor" in professional field 4.3. "Biological Sciences", doctoral program "Biophysics".

28. 08. 2023

Sofia

Signature:

Prof. Iana Tsoneva, DSc