REFEREE REPORT

from Associate Professor Albena Jordanova, PhD,

Sofia University "St. Kliment Ohridski", Faculty of Medicine,

Chemistry and Biochemistry, Physiology and Pathophysiology Department

of PhD Thesis

Professional field: *4.1 Physical Sciences*

Scientific specialty: *Biophysics*

Author: Desislava Henri Lazarova

Doctoral program: unsupervised on an individual plan

Department: Condensed matter physics, Faculty of Physics, Sofia University "St. Kliment Ohriski"

Title: *„Contrast-enhanced magnetic resonance techniques of pathologies, based on cellular redox-imbalance"*

Research consultants: *Prof. Rumiana Bakalova, PhD, DSc and Assoc. Prof. Genoveva Zlateva, PhD*

The present PhD Thesis of Ass. Prof. Desislava Anri Lazarova analyses current problem related to the study of the redox status of tissues and organs in *in vivo* and *in vitro* conditions. For this purpose, for the first time, cyclic nitroxide radicals in combination with nitroxide-enhances magnetic resonance techniques: magnetic resonance tomography and imaging (MRI) and electronic paramagnetic resonance (EPR) were used. These techniques are with high sensitivity and resolution. It is known that cell redox signalisation regulation is one of the factors for maintaining the cell homeostasis in normal range. Healthy cells and tissues are characterised with low concentrations of the reactive oxygen species (ROS) and a constant level of the reducing equivalents. Therefore, the changes in the optimal redox balance in the cells, registered by redox imaging, are an indication for different disorders, such as neuro-degenerative and autoimmune diseases, cancerogenesis, inflammation processes, etc.

The PhD Thesis is extremely diligent and gives an excellent insight into the research done by the PhD student. It consists of 95 pages, 39 figures and 1 table. The reference list provides 144 mainly foreign literature sources.

In the *Literature Review* an in-depth analysis of the problem under consideration is made, detailing the physical principles of the main magnetic resonance imaging techniques (MRI and EPR), the cellular redox status, the main endogenous sources of reactive oxygen species (ROS) and the mechanisms of their disposal. A special attention is paid to the use of redox sensors and nitroxide-enhanced magnetic resonance techniques for the visualization and analysis of the redox status and the levels of oxidative stress, which is important for the diagnosis and monitoring of various diseases such as Alzheimer's, Parkinson's disease, myocardial infarction, and etc. The *Literature Review* is comprehensive and illustrated so it consistently introduces the reader to the research goal of Desislava Lazarova: developing contrast-enhanced magnetic resonance methods to visualize pathologies related to cellular redox status disorders. The tasks are summarized in 4 main areas, including the study of the effect of endogenous oxidants and reductants on the nitroxide-enhanced MRI/EPR signal of the model cell-free systems; application of the nitroxide-enhanced EPR for visualization and assessment of the cellular redox status of isolated cell lines under *in vitro* conditions; visualization of the tissue redox status in mice with experimental models of hypercholesterolemia and parkinsonism. The clearly formulated tasks provide a basis for the presentation and interpretation of the results obtained in the dissertation.

Materials and Methods section contains 13 pages, in which a detailed description of the materials and reagents used, as well as of the preparative and analytical methods for visualization and analysis of the redox status of the cell lines and tissues tested was provided.

The results and their discussion were described on 38 pages. As a result of the precisely conducted experiments, the effects of endogenous oxidants on the nitroxide-enhanced MRI signal of model cell-free systems, potassium superoxide and hydrogen peroxide, are studied, using the mito-TEMPO spin probe, which penetrates the cellular and mitochondrial membranes. Control experiments are also made showing the variations of the EPO signal of mito-TEMPO in the presence of various reducers, ascorbate, NADH, NADPH and glutathione.

The *in vitro* experiments by EPR spectroscopy of isolated cell lines include assessed and compared the redox status of normal and 3 types of cancer epithelial cells, isolated from the lining of the colon with varying degrees of proliferation and differentiation. The results confirm the presence of oxidative damage in the cancer cells due to irreversible impairment of their redox status. In addition, the nitroxide-enhanced EPR signal with mito-TEMPO shows definitely that the increase in the degree of cancer cells differentiation and invasiveness leads to an increase in the overproduction of ROS. The reducing equivalents levels in the cancer cell and their correlation with the nitroxide-enhanced EPR signal are also proved with 4 other conventional analytical tests (including analyses of hyperoxides, cytosolic and mitochondrial super-

oxide, and analysis of the total reducing capacity of the biological samples). These observation is a prove for the adequate application of mito-TEMPO as suitable redox sensor for the study of cells redox status.

In the dissertation thesis presented for review, especially valuable in the application aspect are the studies related to the visualization of tissue redox status in kidney damage in mice, using MRI and mito-TEMPO as a contrast substance. Kidney dysfunction is triggered by a diet leading to hypercholesterolemia and glomerulosclerosis. Moreover, the experiments include a group of mice that, after a 15-day cholesterol diet, have been treated with the anti-lipid preparation cholestyramine that binds to the bile acids and inhibits their reabsorption in the small intestine. The nitroxide-enhanced MRI signal of kidney of mice on normal diet, cholesterol diet and cholestyramine (after injection of mito-TEMPO) showed with no doubt that the higher intensity of the MRI signal in the kidney of mice with hypercholesterolemia was due to elevated ROS levels in kidney tissues compared to that of healthy mice tissues. The effect of cholestyramine administration was recorded by MRI, which shows a decreased intensity of the MRI signal. In addition the kinetic curves of nitroxide-enhanced MRI signal in whole kidney, kidney medulla and cortex were analysed. The results demonstrate the local effect of cholestyramine, which completely eliminates the action of cholesterol in the medulla but not in the renal cortex of the test animals, suggesting a higher oxidative capacity and risk of pathological changes in the cortex.

In addition, to confirm the efficacy of mito-TEMPO (passing through the blood-brain barrier) as a convenient redox sensor in imaging, the tissue redox status in the cortex and the dopaminergic region of the brain of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -treated mice with an experimental model of parkinsonism was visualized. It is found that the kinetic curve of the MRI signal in their dopaminergic brain region following injection of myth-TEMPO differs from the one detected in the control group. In contrast to the dopaminergic region of the brain, the registered kinetic curves in the cortex of the brain showed slight differences between control and MPTP-treated mice. These experiments confirm the changes in the redox status of tissues in the dopaminergic region of the brain, which is most probably due to the neurotoxic effects of MPTP and degeneration only of the dopaminergic neurons.

The results obtained in the dissertation demonstrate the possibilities of high-resolution redox imaging and direct detection of overproduction of ROS in living cells and tissues as a promising new approach in imaging diagnostics and clinical practice.

The scientific contributions from the described studies are summarized in 3 main directions with markedly application character in the imaging diagnostics of kidney damage, using the mito-TEMPO spin probe.

The results of the dissertation work have been published in six scientific papers. Four of the publications are in journals with impact factor, with two of them, where the dissertant is the first author, are submitted for publication. The total impact factor of the publications is 8,904. There are 73 citations of PhD publications. The results are presented at 4 scientific forums in Bulgaria and abroad.

The presented Synopsis of the PhD thesis by Dessislava Lazarova is excellent, fully corresponds to the content of the dissertation and gives full information about the conducted experiments, the results obtained, the discussion and the analysis of the research.

I have the following remarks and questions to the student regarding the PhD Thesis:

1. The enzyme xanthine oxidase catalyzing the conversion of hypoxanthine to uric acid in the purine degradation is described as hydroxylase (page 24 in the dissertation), but it is oxidoreductase with EC 1.17.3.2. This inaccuracy is partially corrected later in the text where its enzymatic action is described correctly.
2. It is known that neurodegenerative diseases are characterized by characteristic β-amyloid plaques in the brain resulting from the accumulation of amyloid proteins, as well as the so-called tau-proteins. Did the beta-amyloid plaques be observed in the experimental model of Parkinson's disease in mice that have dopaminergic neurodegeneration (p.36) used in the dissertation, and are there clear indications that treatment with MPTP is a suitable dopamine neurotoxin inducing degeneration of dopaminergic neurons in Parkinson's disease
3. EPR spectroscopy of *in vitro* cell suspensions is described on page 43. On what the length of the measurements depends on, as it is stated in the text that "the measurements are carried out according the protocol described above for 1-360 minutes (depending on the type of sample)"?
4. Why do you use different inhalation anesthetics, isofluran in the models of hypercholesterolemia, and uretane for the models of parkinsonism, for the MRI experiments with animals in *in vivo* conditions (page 44)?

CONCLUSION

From the research done and analyzed I can summarize that Desislava Lazarova is a fully trained young scientist for conducting in-depth scientific research, possessing the necessary experimental skills and ability to discuss the results obtained and to compare them with literary sources. With the submitted publications and participation in scientific forums, she meets the requirements of the Law on the Development of the Academic Staff in the Republic

of Bulgaria and the Regulations for its Application at the Sofia University "St. Kliment Ohridski".

All this gives me a reason to recommend to the disciplinarian **Desislava Henri Lazarova** to be awarded the educational and scientific degree "doctor" in the professional field 4.1 Physical Sciences, Scientific specialty: Biophysics.

25.03.2019 г. **Referee**:

/Assoc. Prof. Albena Jordanova/