

Dr hab. Aleksandra Jung, prof. AGH  
AGH University of Science and Technology  
Faculty of Physics & Applied Computer Science  
Department of Medical Physics and Biophysics  
30 Mickiewicza Ave.  
PL-30 059 Krakow, POLAND

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#### Review

of the Ph.D. thesis of Ms. Hanieh Karimi entitled

***Characterization of melanoma cell spheroids by means of imaging techniques and positron annihilation lifetime spectroscopy***

Supervisors: Prof. dr hab. Paweł Moskal, Prof. dr hab. Ewa Stępień

#### *General description and content-related evaluation of the thesis*

The dissertation consists of fifteen chapters grouped into 4 parts: Theoretical background, Materials and Methods, Results, and Discussion. They are preceded by the abstract and list of abbreviations. The thesis was written on 94 pages altogether. The structure of thesis conforms generally to principles and requests to the structure of scientific thesis.

The Author declares that the main objective of the dissertation was to evaluate the three-dimensional (3D) spheroid characteristics using imaging techniques and examining the positron annihilation lifetime spectroscopy (PALS) as a novel biomedical approach to identify the cancer malignancy level.

The specific main goals of the Ph.D. thesis were as follows:

- The application of microtomography for imaging of small tumor-like structure composed from malignant and non-malignant melanoma cancer cells-spheroids.
- Evaluation of the glucose and oxygen uptake rate distribution in melanoma spheroids with different degree of malignancy.
- Determination of positronium properties in experimental melanoma models.

The thesis is focused on validation whether the measurement of orto-positronium lifetime in 3D melanoma spheroids from two melanoma cell lines is a promising biomarker for the assessment of the degree of cancer malignancy or not. To check this, firstly, the possibilities of using 3D cultures to bridge the gap between *in vitro* and *in vivo* test are verified, secondly, the possibilities of new research techniques such as microtomography and positron annihilation lifetime spectroscopy (PALS) are tested to characterize the physical properties of spheroids and to determine the mean lifetime of positronium in melanoma spheroids with different malignancy rates. Additionally, the rate of glucose and oxygen uptake are investigated, both important for understanding carcinogenesis process and developing new therapies. The topic of this thesis is current and relevant in the context of up-to-date

research and development of new diagnostic and therapeutical tools used in medicine due to achievements in physics.

Along the performed investigations, the Author had the opportunity to familiarize herself with biological (cell culture) and physical (direct measurements) research. She worked in a large research group contributing to its success represented in the form of 6 publications in prestigious journals during 2020-2021, implementing and improving her performed tasks during this time, which has been presented in two publications in which she is the first and second Author respectively.

As mentioned above, the dissertation is divided into 4 parts: Theoretical background, Materials and Methods, Results and Discussion, preceded by Introduction.

The first chapter is dedicated to a short introduction to the subject. It is concise and at the same time comprehensive, it contains the required information in the field of the state-of-the-art supported by relevant citations.

The part titled Theoretical background contains the basics, necessary to understand the effects of the Author's research, her motivation and directions of the study development. First two out of five chapters are dedicated to biological issues, sufficiently described, such as: melanoma, cell culture related aspects, differences between mono layer cell culture and 3D cell culture, problems related to glucose uptake and hypoxia. Next two chapters are related to physical measurement methods: X-ray microtomography and positron annihilation lifetime spectroscopy (PALS). In the first case, the Author focused on the comparison of micro-tomography and computed tomography (CT), and practical aspects related to measurements, it should be trusted that although not included in the work, the physical basics of the operation of both devices are well known. The basics of the second applied technique are described in more detail starting with the positron and positronium description, including the decay of the most common source ( $^{22}\text{Na}$ ), through annihilation phenomenon up to the Tao-Eldrup model. This model could have been characterized in more detail, especially in regards of its approximations. Theoretical considerations are ended with the briefly presented three main goals of the thesis, listed together along with the main steps of this research.

The second part is focused on Materials and Methods. It includes descriptions related to protocols of cell culture, hanging drop method, biomechanical staining techniques, determination of glucose distribution and hypoxia regions and micro-CT imaging in the first two chapters. Schematic drawings prepared by the Author make it easier to get acquainted with the consecutive steps of the listed procedures. Third chapter of this part contains details on PALS technique. In particular, the procedure of spheroids forming and geometry of the measurement system with details regarding the radioactive source and the holding system is described here. The fourth chapter is dedicated to technical details of data analysis and image processing, in particular: charge and amplitude computation, positronium lifetime calculation, and gain calibration.

Results are presented in the third part of this dissertation. The first chapter of this part contains information on biochemical and imaging characteristics of spheroids, the viability of cells in a single spheroid, and their fluorescence images. Ultimately, 3D micro-CT images of spheroids formed by two kind of melanoma cells are visualized. In the context of this laconic and short chapter in relation to the goals set, it is hoped that this research will be continued, and the Author managed

to overcome the first difficulties in selecting appropriate parameters enabling the visualization of spheroids with the use of micro-CT. The second chapter is focused on glucose distribution and determination of hypoxia region in spheroids. Both processes are important for testing new anticancer therapies. The third chapter is dedicated to results obtained by means of PALS technique. First, spheroids cells were characterized, then the mean lifetime of o-Ps atoms in those spheroids was established for two different cell lines characterized with different degrees of malignancy. The presented results are important and provide a starting point for further research.

The fourth and last part of the thesis includes Discussion and Conclusions. In the Discussion section, the most important achievements of the work are synthetically presented in comparison to other studies, and Conclusions clearly refer to the objectives of the work demonstrating that they have been achieved. The Author correctly interpreted the results and used available adequate literature in the discussion.

The bibliography contains 131 items mainly published in last few years, moreover, when necessary, the Author cites older publications.

It is a pity that particular steps of the study were not described in more details, it should have been emphasized that the Author showed only the final result of refining the relevant research protocols, which yet consisted of many intermediate steps. Not only commercial devices were used in the research but also own solutions which eventually required much more time for the appropriate measurement conditions to be defined. Applied methods are unique and the PhD student showed the required versatility in interdisciplinary work - she had to master both the skills of cell culture, but also precise physical measurements and their analysis.

Although the thesis is well-structured and generally well written, the experiment and analysis performed by the Author are mostly clearly described, and the results formulate essential contributions to the field of development of new methods identifying the cancer malignancy level, the Author did not avoid a few minor errors and ambiguities in the thesis. Comments and questions that arose during the Ph. D. thesis reading are listed below:

- The fundamental physical principles of the microtomography are missing, especially the principle of X-ray production and tube characteristic.
- The arrangement of figures and tables in relation to the related text is not always optimal, the figures should be as close as possible to the related text and should not be placed at the end of the chapter if possible.
- Figures and tables are not always referenced by literature in the descriptions. Even if the Author prepared them herself, it was done on the basis of the literature (e.g. Fig. 3.1, Tab. 5.2)
- Incomplete units in Tab. 5.1.
- The physical quantities used in the formulas should have units presented.
- Tao-Eldrup model could be described more in detail, especially its assumptions and limitations depending on application.
- The description of the cell culture should be more precise - how many times was it prepared and for what experiment?

- Excerpts from the Author's own article [79] should be highlighted and appropriate citation added.
- If any process is repeated, there should be information on the number of repetitions, if not the measurement uncertainty should be estimated on the basis of measurement accuracy.
- There is no information regarding analysis of the normality of the distribution of the examined features and the type of *t*-test used (Chapter 10.1). Did the number of measurements/samples allowed for the use of the parametric test?
- Illegible descriptions in figures (e.g. Fig. 10.1).
- Differences visible in Fig. 10.2 should be further explained.
- There is no assessment of the accuracy of the exponent fit (Fig. 10.7).
- Fig. 11.2 - it is not clear why comparison includes only day 7<sup>th</sup>.
- Data presented in Fig. 11.2 and Tab. 11.1-2 differ from those in the Author's own article - contain more or less cases?
- Are the differences presented in Table 11.3-4 significant?
- Lack of uncertainty in Fig. 12.3.
- How was  $D_i$  in the eq. 12.2 estimated?
- Occasional linguistic, grammatical (correct tense) and punctuation errors and typos.

#### *Final conclusion*

Despite some criticism stated above, the dissertation of Ph.D. student Hanieh Karimi describes very interesting and valuable research work towards finding biomarker for the assessment of the degree of cancer malignancy. In my opinion, the doctoral dissertation contains original results which show promising directions for practical implementations of determination of positronium lifetime in melanoma spheroids for grading its malignancy and should encourage other researchers to follow this promising path. This work contributes significantly to the study of malignancy grading reflected at the molecular level that can be probed by using the PALS method, and is important in the general context of research into new diagnostic and therapeutic methods. The previously listed shortcomings do not significantly affect its scientific level and my final positive assessment.

I conclude that the thesis reaches standards of quality required for doctoral thesis and meets the requirements of the Act form of academic degrees and academic titles and degrees and title in art and I make proposal to assume it and admit it to public defense. Therefore I recommend for admission of Ms. Hanieh Karimi to further stages of the procedure towards the degree awarding of Ph.D. in physics.

*Alenard Jurf*