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REVIEW

of a PhD thesis of Karthika Kappalakandy Valapil

"ITO microelectrodes and microelectrode arrays for the analysis of cell cultures and biomedical applications"

The doctoral dissertation of Karthika Kappalakandy Valapil, entitled "ITO microelectrodes and microelectrode arrays for the analysis of cell cultures and biomedical applications" was carried out at the Institute of Physical Chemistry, Polish Academy of Sciences under the supervision of dr hab. Martin Jonsson-Niedziółka, prof. IPC PAS and dr inż. Emilia Witkowska-Nery.

Cell cultures are one of the most useful tools in contemporary science. Their application range from basic studies in biology and biochemistry to testing new drugs and developing organ-on-a-chip devices. The importance of cell cultures arises mainly due their cost benefits, as well as ethical superiority over animal studies. However, to make full advantage of a cell culture experiment, one must be able to control cell proliferation and measure its metabolic activity. A number of tests, mainly optical, have been developed for that purpose and are now routinely used for all types of cell cultures. On the other hand, electrochemical methods, while widely used in analytical chemistry, seem to be underrepresented in the analysis of cell cultures. Scientific research done by Karthika Kappalakandy Valapil is an important step towards expanding the application of various electroanalytical methods in the field of cell culture monitoring. It seems that the most important aspect of her PhD thesis was to develop new electrochemical tools for researchers working in this field. In this review, I will present my opinion on how well she did in achieving this goal. I will also assess the editorial layout of the doctoral dissertation and the overall scientific achievements of Karthika Kappalakandy Valapil.

Assessment of the layout, language and editorial layout of the dissertation

The thesis starts with a list of Author's publications, abstract (in English and Polish), list of abbreviations and table of contents. In the abstract, the aim of the research is given, as well as relatively detailed description of performed experiments.

The main part of doctoral thesis has typical layout and it consists of four main chapters, one of them being Introduction section, while three consecutive chapters contain results of experimental work. Each of these chapters contains subsections describing chemicals used in the experiments, methodology, obtained results and their discussion, as well as conclusions. In chapter 4, describing mediators for glucose monitoring, this structure is used twice, thus in my opinion this chapter should be divided into two separate chapters, each describing work done on a different type of redox mediator. The thesis ends with a summary, future outlook (a very important chapter, rarely found in MSc and PhD theses), one appendix and a list of 232 cited literature references.

The introduction provides some basic information on cell cultures and methods of their analysis. Next, the possibility of employing electroanalytical methods for cell cultures monitoring is discussed in detail, including basic information on electrochemical biosensors and their miniaturization, materials for electrodes and electroanalytical techniques used in the study. The information provided in this section introduces the reader to the subject of the research in a very comprehensive way. Only the topics relevant to the dissertation were presented, which allowed to maintain the correct proportions of the entire thesis.

The thesis is written in the correct language and the number of typos, incorrect wordings and linguistic errors is very small.

Substantive evaluation of the dissertation

In the first project described in the thesis, microelectrodes and microelectrode arrays were developed. Initially, platinum wire electrodes were considered, however after some experiments, this idea was dropped due to their poor performance. Despite trying several methods of electrode fabrication, it was impossible to obtain Pt electrodes of desirable reproducibility and electrochemical characteristics. The story was much different in the case of ITO electrodes, with successful use of CO_2 laser cutting for the preparation of structures from this material. Two techniques were employed, namely direct patterning and in-situ stencil fabrication. Fabricated electrodes were thoroughly characterized using a vast array of microscopic and electrochemical methods. ITO electrodes exhibited stable and reproducible electrochemical response. It was found that direct laser patterning allows for preparation of smaller electrodes, albeit with significant edge damage, while laser stencil ablation was more suitable to manufacture larger electrodes with no edge damage. This two-way approach allows broader range of ITO electrodes that can be prepared using popular CO_2 lasers, based on a particular needs of an analytical process. As a result of this first project, Author developed tools for herself, but also for other researchers, that are invaluable for various biosensing applications.

Second project shows such application: ITO electrode arrays, fabricated using method described above, were applied for monitoring of cell culture growth. Device used in this study consisted of eight round microelectrodes arranged in a circular fashion to ensure symmetrical distribution of electric field. Electrochemical impedance spectroscopy (EIS) was used to assess cell growth and adsorption on the electrodes. As expected, upon increasing cell density, impedance values were increasing. Upon reaching confluence, electrodes were treated with trypsin solution, which was clearly visible on EIS results as an impedance decrease. Similar results were achieved for two

different cell lines. It can be concluded that this project shown great potential of ITO microelectrodes and electrochemical impedance spectroscopy for cell cultures monitoring. One might wonder why this successful study wasn't continued e.g. towards analysis of drug interactions with cell lines. My feeling is that some very interesting results could be achieved by further exploring the potential of ITO/EIS system.

Next two project were concerned with synthesis and application of redox mediators for glucose detection. First, synthesis of a hydrogel modified with polypirydyl osmium complex was attempted. It is clear from the thesis that a lot of effort was put into repeated tries to obtain this compound. Reaction progress was monitored using several analytical techniques. Various approaches to the synthesis were tried, with total synthetic procedure times approaching 30 days. Despite all this, desired redox active metallopolymer was not produced. However, a sample of this compound was donated by Prof's. Donal Leech group (National University of Ireland, Galway). Using this sample, glucose sensor was prepared and tested. It was shown that oxidation current is proportional to the glucose concentration in the 0-10 mmol range. As Karthika Valapil admitted in the Conclusions to this section, one of the possible reasons for unsuccessful synthesis of osmium-based redox polymer is "lack of our experience in this field". While I'm certainly not in the position to advise the Author, I believe that outsourcing the synthesis of a redox metallopolymer earlier and devoting the saved time to more detailed research on electrochemical glucose determination would be beneficial to the thesis.

In search for suitable redox mediator to be used in connection with ITO electrodes, Author turned to study of Prussian blue analogues. Total of 14 different reactant combinations were investigated. Flash light sintering on ITO plates was employed as a synthetic method, with 7 of attempted reactions showing positive results. Unfortunately, the stability of most synthesized redox markers was not better, as compared to other synthetic methods. Among the most promising compounds tested in this study were: potassium-cobalt hexacyanoferrate and potassium-nickel hexacyanoferrate. Somewhat surprisingly, only unmodified Prussian blue, obtained using flash light sintering, was studied in detail as a redox marker for hydrogen peroxide determination, with calibration curve prepared in 0-20 mM concentration range. It should be mentioned that these measurements were carried out using glassy carbon electrodes, not ITO. Moreover, H_2O_2 determination could hardly be called a "biomedical application" of a modified electrode. Both of these facts are in contrast to the title of the thesis, which mentions application of ITO microelectrodes for biomedical measurements. Generally speaking, however, this section contains some important data that can be of interest for further development of glucose sensors. Uncharacteristically for this thesis, results are described in slightly chaotic manner, making this fragment somewhat hard to follow.

To sum up, it should be stated that the reviewed PhD thesis has an aspect of scientific novelty. Author has carried out systematic and detailed studies on the fabrication of ITO electrodes of various sizes. These devices were then successfully applied for monitoring of cell culture growth using electrochemical impedance spectroscopy. Work on the synthesis of mediators for glucose determination, while less successful, resulted in a detailed study of synthesis and application of Prussian blue analogues which can be of interest to researchers working on electrochemical biosensors. Performing all the described work required, in addition to the perfect

knowledge of electrochemistry, also an expertise in organic synthesis as well as engineering skills.

I rate the quality of the research carried out by Karthika Kappalakandy Valapil and the manner of their description highly. However, the reviewer is also obliged to point out the shortcomings of the work to the Author. Therefore, below I am listing my comments to this dissertation, asking the PhD student to comment on the following issues during the defense:

- Abstract and many times throughout the thesis: "... cost-effective analytical tool..." – the statement about low cost of ITO electrodes is repeated quite often in the thesis, however actual cost of fabricated electrodes is not given. Could Author provide a price for any of the ITO electrodes described in the thesis? Of course, the unit price strongly depends on the scale of production, thus assumptions are necessary as to the number of electrodes produced in one production batch.

- p. 27: description of working mechanism of redox hydrogels, especially fragment on wiring the reaction centers of immobilized enzymes to the electrode, seems to be more fitting to third-generation enzymatic sensors, while those are second-generation sensors. Could Author clarify this?

- p. 34: it seems that Pt microelectrodes had an internal electrolyte (1 M KCl solution) between Pt and Cu wires. Why direct connection of these two wires was not used instead?

- p. 52: "Single electrodes were used repeatedly over several months without a deterioration in signal over time" – results of these measurements were not shown in the thesis. How were the electrodes cleaned between the experiments?

- p. 67: why glassy carbon electrodes were used for glucose sensing with osmium-based redox hydrogel, and not ITO electrodes?

- p. 79: results of glucose sensing measurements using osmium-based redox hydrogel synthesized at IPS, PAS should be shown and compared to analogous measurements performed using mediator samples obtained from NUI Galway.

- p. 83: why glassy carbon electrodes were used for H_2O_2 sensing with Prussian blue, and not ITO electrodes?

- p. 89: "...current values were decreasing with increasing H_2O_2 concentration" – current values are increasing with increasing H_2O_2 concentration, as it can be seen in Fig. 46.

- p. 91: synthesis of Prussian blue analogues on titanium substrates is mentioned in Conclusion section, however it is not discussed in Methods section or in Results and discussion.

Below I list some minor remarks to the dissertation that the PhD student does not need to refer to during the defense:

- p. 79 and Fig. 40: current originating from mediator, not "glucose oxidation current", was measured.
- p. 83: spelling of "μpulses" is rather interesting.

The list of publications by Karthika Kappalakandy Valapil contains two articles in scientific journals and one book chapter. It seems that only one of these publications is related to the subject of the thesis, therefore overall scientific achievements of PhD student can be considered as modest, although the high rank of journals should be noted.

I hereby state that the reviewed dissertation meets all of requirements – both formal and customary – for doctoral theses as specified in Act of July 20, 2018 - Law on higher education and science (Journal of Laws of 2023, item 742 as amended) and I submit to the Scientific Council for Chemical Sciences of Institute of Physical Chemistry, Polish Academy of Sciences to accept the dissertation and admit Karthika Kappalakandy Valapil to further stages of the procedure for awarding a doctoral degree.

Sincerely,