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Review of doctoral dissertation

Karthika Kappalakanda Valapil, M.Sc.

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

The basis for this doctoral dissertation review is a letter from the Deputy Director for Scientific Affairs of the Institute of Physical Chemistry, Polish Academy of Sciences, prof. Jacek Gregorowicz, dated April 11, 2024 (RPW/15308/2024 N). The doctoral dissertation submitted for evaluation was prepared in the Institute of Physical Chemistry, PAS, in the Charge Transfer Processes in Hydrodynamic Systems group. The doctoral dissertation supervisor is Prof. Martin Jönsson-Niedziółka and the auxiliary supervisor is Dr Emilia Witkowska-Nery. The work was partially funded by the National Centre for Research and Development, within the grant LIDER/38/0138/L-9/17NCBR/2018, entitled "*Three-dimensional substrates with an integrated multi-electrode measurement system for applications in cell cultures and pharmaceuticals*", led by Dr. Witkowska-Nery, one of Ph.D. candidate supervisors.

The doctoral dissertation explores the significance of electroanalytical tools in biochemical studies, particularly focusing on cellular processes. These tools offer significant advantages for various process monitoring due to their high sensitivity, specificity, and real-time detection capabilities through precise measurement of electroactive species within complex biological systems. Electrochemistry offers cost-effective, simple-to-use, on-site, and rapid detection of low concentrations of biomolecules, such as neurotransmitters and metabolic intermediates, facilitating insights into cellular function and signaling pathways. Since electroanalytical methods are minimally invasive and can be integrated with microelectrodes, they tempt with an in vivo monitoring potential with high spatial and temporal resolution, simultaneous multianalyte detection, and more, providing comprehensive data on cellular environments and biochemical interactions. Moreover, as the Author of the dissertation notes, electrochemistry offers an accurate and ethical alternative to animal testing.

It should be highlighted that introducing and developing new ideas and technologies in the biosensors field, postulated as this dissertation's fundamental aim, requires vast and interdisciplinary knowledge related to chemical sciences, materials engineering, biochemical engineering, biotechnology, signal processing, and more. Thus, it should be considered a notable and demanding challenge for the Ph.D. candidate.

Karthika Kappalakanda Valapil's doctoral dissertation was prepared in the form of a manuscript, presented on 114 pages and characteristic of doctoral dissertations. The manuscript contains 232 independent citations, the vast majority of which refer to publications in JCR journals, published in the author's research area in the last decade. These references are used properly throughout the text, however, it would be very beneficial if a few more references were provided when the Author is making a decisive statement or conclusion (examples below). The Author pays attention to the editorial aspect of the dissertation. The text contains few grammatical errors, while the graphics illustrating described phenomena and experiments are prepared with the required care.

The manuscript is divided into four chapters. The Introduction section presented first constitutes a theoretical background to familiarize the reader with the fundamentals and the state of the art of the investigated field. Next, three consecutive chapters may be considered as individual research endeavors for the author of the dissertation. While not directly connected at first glance, the studies contribute to the development of different components for the final device, namely: (i) fabrication of versatile indium tin oxide (ITO) microelectrode arrays, (ii) capability of impedimetric studies for cell cultures characterization and (iii) signal enhancement ability by Os metallopolymers and Prussian Blue (PB) analogs. While the manuscript structure is clear, it misses a clear statement from the Author regarding the primary goal of the thesis and research hypotheses to be verified during her studies. An important explanation regarding the selection of specific research tasks and individual challenges to offer new insights into the development of diagnostic tools was presented only in the last chapter of the manuscript.

As stated beforehand, the first chapter of the manuscript provides a valuable and clear introduction to different aspects of this interdisciplinary study. As an electrochemist who is not experienced in operation with cell cultures, I highly value the educational value of the presented discussion related to types of cell lines, their mortality, dimensionality and the consequences these aspects bring in the cell culture analyses. The chapter outlines different techniques used for signaling and neurotransmission studies, such as ELISA, HPLC, and NMR, and highlights the prospects resulting from the use of electrochemical techniques.

Next, the Author discusses the background of her choice of electrode materials, focusing on platinum and indium tin oxide (ITO). In my opinion, this selection requires further discussion in terms of the advantages and disadvantages of these two materials compared to gold or carbon-based (BDD, GC, LIG, graphene, etc.) electrodes, in particular regarding their environmental stability, micro-sized fabrication capabilities, and antifouling properties. A deeper discussion of the state of the art in this particular aspect would be helpful for the reader. An important aspect discussed here is the capability of different laser-induced patterning techniques for microelectrode array fabrication, highlighting an economic aspect of this approach originating from the utility of common CO_2 lasers. Next, the Author introduced various electroanalytical methods used in biosensing. While the chapter may be valuable for non-electrochemists, it may be considered outbalanced as all of the DC techniques are characterized in 1.5 pages of text, while nearly 6 pages were dedicated to electrochemical impedance spectroscopy (EIS). The majority of electrochemical studies in this dissertation present voltammetry results while only Figs 25-27 refer to impedimetric results. In my opinion, a better introduction to electrical cell-substrate impedance sensing (ECIS) instead of EIS fundamentals would be of benefit to the reader's understanding of the results presented within Chapter 3. Chapter 1.4 discusses the fundamentals and state of the art regarding the electrochemical mediators for glucose biosensing, introducing the concept of 2nd generation of biosensors with osmium-based redox hydrogels and electrodes modified with

PB analogs. Here, the reader might find a direct mental leap from cell cultures to glucose biosensing confusing, without at least pointing out a key significance of glucose monitoring for cell metabolism or growth conditions research and the modus operandi of glucose biosensors.

Chapter 2 focuses on the fabrication and characterization of the Author's own microelectrodes. Among the studied Pt and ITO electrodes, the former ones appeared to fail in delivering reproducible results, an aspect pointed out and honestly discussed by the Author, and seems to be related to the fabrication procedure, uncontrolled capillary etching, and impurities. The Author mentions that the optimization of laser ablation was a trial-and-error process, yet the lack of details and statistics makes it impossible to try and reproduce these results and understand the origin of the difficulties discussed in the results section. On the other hand, I consider the fabrication and characterization of ITO microelectrodes one of the main achievements of the Author in this dissertation. Utilizing microscopy and spectroscopic tools she reveals high-quality micron-scaled ITO patterns, discussing not only morphology and chemistry but also the resulting charge transfer homogeneity and presence of boundary effects, which may alter electrode reproducibility in particular in the case of the smallest, 25 µm-wide microelectrodes. This was further confirmed by statistical analysis of performed voltammetry results in the presence of model redox species. Next, in Chapter 3 Author utilizes the previously fabricated ITO microelectrode arrays to successfully track HepG2 cell growth and subsequent detachment upon trypsin injection. These measurements were performed upon evaluation of impedimetric characteristics of the studied system and identification of the frequency range capable of effective differentiation of the studied processes. In these chapters, the Author proves her competencies in the fields of materials engineering, electrochemistry, and microbiology, contributing valuable new and original research to the existing biosensors state of the art.

Finally, in Chapter 4 Author explores two independent routes for charge transfer mediation aiming for improved glucose biosensing. The first approach utilizes poly(vinyl imidazole) (PVI) polymers, functionalized with cis-$Os(N\text{-}N)_2\text{-}Cl_2$ redox centers. This Chapter testifies to the organic synthesis skills of the Ph.D. candidate, as she was capable of preparing different PVI-bound osmium complexes and confirming their purity using NMR and mass spectrometry techniques. The electrochemical characterization of these complexes appears to be demanding, however, with limited stability over multiple polarization scans. This effect consequences in the redox process's irreversibility and/or the appearance of secondary redox peaks over time. Moreover, the Author reports that the coupling reaction for some of the studied compounds may last as long as 21 days. As a result of the know-how obtained by numerous tries and characterization methods, a conclusion was drawn that further modification of the synthesis procedure is needed. Yet, the potential of Os-PVI metallopolymers in glucose sensing was presented based on the hydrogel fabricated in cooperation with the National University of Ireland (prof. Leech group). Next, the Author studied multiple Prussian Blue analogues, synthesized by a flash light sintering (FLS). It is a novel fabrication approach, that fits perfectly into the general vision of cheap and easy-to-fabricate electrodes. Moreover, according to the Author's claims, FLS should offer higher PB stability during repetitive CV scans. Indeed, the author confirmed in her research, that in particular, a PB analog made of $NiCl_2$ and $K_3Fe(CN)_6$, is capable of providing fairly reproducible CV scans for up to even 50 cycles, concluding the utility of the original FLS approach.

The doctoral dissertation of Karthika Kappalakanda Valapil, M.Sc., which was submitted for evaluation, is written following standards of scientific research. Nevertheless, while studying the dissertation, I crossed

upon some controversial descriptions, either not clear or incomplete. Thus, I ask the Author to clarify the discussion regarding the following topics:

1. The Author presents valuable information regarding the reproducibility of the $FcDM^{0/+}$ peak currents at ITO microelectrodes in Fig. 20, yet without further detail on redox process reversibility. The Author is requested to present CV peak-to-peak separation ΔE similarly. Have you performed any studies with a redox probe characterized by an inner sphere electron transfer mechanism, such as $[Fe(CN_6)]^{4-/3-}$?
2. Concluding Chapter 2 of the manuscript Author claims that "*single electrodes were used repeatedly over several months without a deterioration in signal over time*". These studies are not presented in the manuscript. The Author is requested to provide results supporting this claim. What were the environmental conditions for electrode exposure?
3. Given that the Author carries out cell culture monitoring with EIS, why no EIS results are revealed also in Chapter 2, during ITO optimization? Please provide the EIS spectra for ITO microelectrodes in the presence of $FcDM^{0/+}$.
4. Within Chapter 3 the impedance was only measured at frequency = 72.1 Hz. How was this value selected? On p. 54 Author states that impedance was measured for frequencies between 10 Hz and 40 kHz, next, on p. 56 she claims that the adherence interaction did not introduce substantial changes in Z'. Yet, no such experiments (or references) are presented in the dissertation. In my opinion, it would be beneficial to present full impedance spectra before and after cell introduction in the system, as well as after trypsin addition. Have you studied how these particular steps influence the open circuit potential value?
5. In the same experiment, Author states that cells were introduced when "*the medium is stabilized*". What is the stabilization criterion? In Fig. 25 the impedance seems to be still dynamically changing before cell addition.
6. Did the Author verify the role of glutaraldehyde crosslinking density on the performance, mass, and charge transport by studied microelectrodes? What is the role of electrolyte pH on the hydrogel stability and the charge transfer process?
7. To what extent argon purging should be considered effective when studying hydrogel coatings immersed in the electrolyte? Is the diffusion rate of the oxygen through the hydrogel coating sufficiently high to justify this procedure?
8. Why were GCEs used in Chapter 4? It is misleading for me which experiments were carried out with GCE and which with ITO. Moreover, GCE is said to be modified with "*carbon nanoparticles and graphite nanoparticles*" (p. 82). Neither the NP chemistry, GCE modification route, nor role in the charge transfer kinetics are explained. Please provide further explanations.
9. The Author discusses the fabrication of fourteen PB analogs, yet limits herself to present only two of them, also stating that some others resulted in unsatisfactory signals. I kindly ask the Author to reveal CV analyses for all the studied PB analogs. I think a valuable visualization could be to show a graph of the relative change of anodic/cathodic peak current (Y-axis) vs consecutive scan cycles (X-axis). Moreover, the PB appears to be pH-sensitive, yet the Author performs her studies in various pH conditions (pH =2.5 in Fig. 42, =7.4 in Fig. 41, 43, 44, 45, and even =5.8 in Fig. 46). Why is that?

10. Glucose sensing in Fig. 46 was carried out at -0.5 V (I assume vs reference Ag|AgCl electrode, as it was not stated directly. Yet, Fig. 47 shows the CV only in the range between -0.3 and +1.3 V. Please provide an extended CV scan, with and without H_2O_2.

The comments and questions listed above do not affect the final, positive assessment of the work of Miss Karthika Kappalakanda Valapil, and do not reduce the cognitive value and originality of the proposed solutions. In particular, I consider the proposed route for successful ITO microelectrode array fabrication and optimization, and then its proof-of-concept use for cell culture monitoring to be a very valuable and original element of the doctoral dissertation, undoubtfully constituting an important scientific accomplishment of the dissertation Author. She has also shown that the ITO electrodes prepared with the use of printing shop infrastructure provide reliable and reproducible substrates for biosensor studies. A second, notable achievement of the Author of this dissertation is the demonstration of FLS as a tool enabling the effective fabrication of PB-modified electrodes that maintain reasonably higher redox process stability upon multiple cycling compared to PB-modified by more common, electropolymerization route. Despite eventually unsuccessful efforts to introduce reproducible Os-PVI metallopolymer-modified surfaces to glucose sensing, the know-how obtained by the Author is unique and will be valued in further functionalization attempts.

Below, I have also stated some minor comments and editorial errors that do not require further discussion on the Author's side:

- It is advised to avoid using abbreviations in the title of the dissertation or the manuscript, in particular without their explanation.
- Page 19. Rs should be considered series resistance, and not solution resistance, in some cases, electrode resistance might be higher than this of electrolyte.
- Explaining the synthesis of osmium-based metallopolymers lacks references to previous works.
- Statements such as "*measurement (...) was relatable to the literature results*" (p. 68) or *"(...) procedures yield unstable PB complex structures*" (p. 84) and few others require confirmation in the form of appropriate references of experimental studied
- The quality of Fig. 41c is suboptimal and sloppy. Similar to Fig. 41a and 41b this graph should present only selected stans: 1st, 10th, 20th, 200th, etc.
- Information about experimental conditions, i.e. electrode type, scan rate, etc. should always be provided in figure captions.

To sum up my review, I can say that the dissertation contains original research results and significant elements of scientific novelty, reaching beyond the existing state of the art. The assessed doctoral dissertation by Miss Karthika Kappalakanda Valapil, M.Sc., entitled: "*ITO microelectrodes and microelectrode arrays for the analysis of cell cultures and biomedical applications*" meets the formal requirements for doctoral dissertations under the Act of July 20, 2018 - Prawo o szkolnictwie wyższym i nauce (Dz. U. poz. 1668 z późn. zm.). Given the above, I recommend to the Scientific Council of the Institute of Physical Chemistry, Polish Academy of Sciences to accept the doctoral dissertation and admit Karthika Kappalakanda Valapil to the next stages of the doctoral conduct.