Wydział
Chemiczny

Prof. D.Sc. Ph.D. Eng Elzbieta Jastrzebska
Faculty of Chemistry
Warsaw University of Technology
Noakowskiego 3, 00-664 Warsaw, Poland
ph.(+4822) 234 7331
elzbieta.jastrzebska@pw.edu.pl

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„Deciphering the role of antibiotic exposure at the sub-Minimum inhibitory concentration on bacterial heterogeneity at single-cell level"
of Ph.D. Candidate M.Sc. Shakeel Ahmad

Ph.D thesis was realized within the Warsaw-4-PhD Doctoral School at the
Institute of Physical Chemistry Polish Academy of Sciences
Department of Soft Condensed Matter Kasprzaka 44/52, 01-224 Warsaw
Supervisor: Prof. dr hab. Piotr Garstecki
Auxiliary supervisor: Dr. Ilona P. Foik

The doctoral dissertation submitted to me for review addresses a very important aspect, which concerns issues related to the improper use of antibiotics and their impact on the growth of bacterial resistance. Suboptimal dosing may not eliminate the entire bacterial population, allowing some cells to survive under conditions of constant, low antibiotic stress, which may promote the development of resistance. How was underlined by Ph.D. Candidate, one of the key factors in heteroresistance is the ineffective and inappropriate use of antibiotics. The studies are often focused on determining the minimum inhibitory concentration (MIC), and the investigation of the influence of the subtherapeutic dosing on individual cell responses is omitted. It should be noted that single-cell techniques used in antibiotic susceptibility testing can provide important insight into bacterial survival strategies and treatment. Within this thesis, Ph.D. Candidate covers the

Politechnika
Warszawska

ul Noakowskiego 3
00-664 Warszawa
www.ch.pw.edu.pl

broadly understood topic above. Ph.D. Candidate aimed to study how pre-exposure to antibiotics below the minimum inhibitory concentration (MIC) affects heteroresistance at the single-cell level during subsequent antibiotic exposures to the same or different antibiotics. Droplet microfluidics has been used in research as a tool to study the sensitivity of individual cells. Without a doubt, the subject of the reviewed doctoral dissertation is important, in the context of the future application of microfluidic systems in biomedical research and diagnostics.

The dissertation submitted for review includes seven chapters: Introduction, Goal of the thesis, Materials, Methodology, Results, Discussion and Conclusions, and References (246 items). Additionally, the doctoral dissertation covers a Table of contents and an Abstract. The entire doctoral dissertation consists of 124 pages. The work was written in English and enriched with charts, tables, and figures. On the editorial side, I rate the work very well.

The first part of the doctoral dissertation is the Introduction. It covers 34 pages. This Chapter characterizes bacteria and their beneficial roles, describes antibiotics, the historical perspective, and their types. The methods of quantifying bacteria and their interaction with antibiotics have also been discussed. This part discusses antimicrobial resistance, the role of reactive oxygen species in antibiotic action, heterogeneity in bacterial populations, and detection of heteroresistance. The introduction is closed on droplet microfluidics as a good solution to study the heteroresistance in bacteria at the single-cell level. I consider the Introduction to the subject of work and the presentation of fundamental issues regarding and familiarization with the methodology that is reflected in the work have been very well covered, but I feel that there are not enough presentations of the newest solutions in the study of the sensitivity of antibiotics used in this research, including the use of droplet microfluidics. A short chapter summarizing the literature part of the work is also missing. The number of references is around 250. This may raise concerns regarding the critical selection of literature in the Introduction and Discussion sections.

The next chapter is the Goal of the thesis, in which the objective of the study was described and the aim of the study was defined. The Ph.D. Candidate aimed to study how sub-MIC antibiotic exposure influences the heteroresistance in individual cells. At this point, I lacked a clearly stated research hypothesis. Please clarify whether you established the hypothesis, and if so, what the hypothesis of your doctoral dissertation is. The following parts of the doctoral dissertation are Materials, and Methodology. It covers 10 pages. Methodology is divided into chapters describing methods of bacterial culture, chip fabrication, droplet generation, and image acquisition and analysis. The next chapter is the Results. This chapter is divided into two sub-

chapters; first, the results for *Escherichia coli* MG1655, and second, for *Pseudomonas aeruginosa* PA14 cells have been presented. In both cases, MIC values for ciprofloxacin and streptomycin were determined in the first stage. Next, the bacteria were pre-exposed to 0.5X, 0.25X, and 0.125X MIC of ciprofloxacin and streptomycin separately, and treated with one of these antibiotics. The graphs showing the resistance profile and dependence of fraction of individual cells that could proliferate (F_RC) and antibiotic concentration (c) were presented for each type of experiment. Based on that several parameters were determined, such as: $(iMIC)_{mode}$ (the most probable antibiotic concentration), $(iMIC)_{start}$ (concentration where the first drop was observed on Gompertz fitting), $(iMIC)_{all}$ (concentration needed to inhibit growth in all droplets completely), p(*i*MIC) (probability density distribution of *i*MIC), and finally, the degree of hetetoresistance (DoH) was calculated. All results have been collected in tables, which clearly indicate whether and how pre-exposure with antibiotics affects the change in the specific parameters and heteroresistance. One of the determined parameters was $(iMIC)_{exp}$ – the concentration that stops growth in all droplets seen with the microscope. *However, the Ph.D* Candidate didn't add any results, images based on which these parameters were determined. Similarly, this results section does not present any photos of the microsystems used, nor the method of docking cells in the droplets (apart from the image presented in the Methodology). I would also like to ask to explain what percentage of droplets were positive, i.e., with docked cells, and whether it was possible to distinguish results for drops with more than one cell? The Ph.D. Candidated noticed that the pre-incubation with sub-MIC doses of antibiotics can increase the MICs of single cells kept in the drop and change the degree of heteroresistance, but the observed dependency is not explicit. In the last part of the dissertation, in the Discussion and Conclusion, the Ph.D. candidate underlined the importance of conducting the research that forms the basis of this doctoral dissertation, and tried to explain the obtained results. This Chapter is divided into "Droplet microfluidics as an excellent tool single-cell study", in which the results for both types of bacteria have been discussed, and "General discussion and conclusion". It should be noted that the Ph.D. Candidate presented a very good discussion of the results.

There is no doubt that the doctoral dissertation has an original aspect to a scientific problem that has been studied. Ph.D. Candidate raises a critical issue concerning heteroresistance, the phenomenon in which a bacterial culture contains subpopulations that exhibit significantly higher minimum inhibitory concentration (MIC) values than the main susceptible population. Undoubtedly, an important aspect of this doctoral dissertation is the approach to studying

heteroresistance, which allows for differentiation at the level of a single cell. For this purpose, a microfluidic system based on droplet generation and single bacterial cell analysis was used. This is an undeniable advantage compared to the tests used to date. As I mentioned before, the Ph.D. Candidate refers very laconically to the description of the operation and fabrication of the microsystem. The microsystem presented in this way is only a tool used in research. The Ph.D. Candidate does not explain the geometry of the system used to generate droplets or justify its use, nor does he present photographs of the microsystems made. I would be grateful for an explanation of these issues during the public defense of the doctoral thesis. Inappropriate use of antibiotic therapy, resulting, among other things, from the heterogeneity of the bacterial cell population, can lead to many complications or ineffective treatment. In his research, the Ph.D. Candidate indicated that sub-inhibitory antibiotic exposure can both enhance and inhibit heteroresistance. It depends on many factors, such as bacteria types, antibiotic class, and antibiotic concentration pre-exposure. Such conclusions were drawn based on two types of cells studied, *Escherichia coli* MG165 and *Pseudomonas aeruginosa* PA14, which were selected in a thoughtful way, and were sub-inhibited with two different antibiotics: ciprofloxacin or streptomycin. The Ph.D. Candidate carefully selected the antibiotics to be tested; ciprofloxacin is a fluoroquinolone, a target of DNA replication, which induces the production of ROS, promotes DNA mutations, and facilitates resistance evolution. In turn, streptomycin, an aminoglycoside, targets protein synthesis with potential ROS generation, DNA mutation, and protein aggregation. Studies using *Escherichia coli* MG165 showed that pre-exposure with 0.5X MIC and 0.25X MIC ciprofloxacin led to a significant increase in heteroresistance during the subsequent antibiotic exposure to both ciprofloxacin (20-fold increase for 0.25X MIC and 0.5X MIC) and streptomycin (15-fold increase for 0.5X MIC). No changes were noticed for pre-exposure with streptomycin. The Ph.D. Candidate concluded that the ROS generation and DNA mutagenic potential of ciprofloxacin are more effective in these cells; however, no evidence (for example, concerning ROS analysis) was not shown in the description. *I* will ask about the comment for that. In turn, changes in DoH were observed in *Pseudomonas aeruginosa* PA14 cells both in the case of pre-exposure with ciprofloxacin and streptomycin and in subsequent exposure with streptomycin and ciprofloxacin, but they appear to be very random. Streptomycin pre-exposure increased the heteroresistance of the population on subsequent exposure to both antibiotics. However, ciproproflaxicin induced more heteroresistance. In some cases, a decrease in DoH was also noticed. In the discussion section of the doctoral dissertation, the Ph.D. Candidate attempts to explain the observed correlations in DoH changes based on the

mechanisms of antibiotic action. However, this is not easy due to the lack of clear correlations. Additional research, e.g., in the field of molecular biology, may help to explain this, but I understand that this was not done at this stage of the work. It would definitely be an added value, if Ph.D. Candidate have tried to carry out the selected experiment to clarify the obtained results. On the other hand, such diversity in the heteroresistance obtained shows how important it is to conduct research at the single-cell level.

The research conducted within this dissertation provides knowledge and tools enabling study on different strains and antibiotic types, and the analysis of the influence of sub-MIC antibiotic exposure on the heteroresistance. To sum up, the findings of this thesis offer a novel perspective on the consequences of suboptimal antibiotic use, regarding how it influences heteroresistance in bacteria at the single-cell level during subsequent treatments. It is doubtful that the research could provide a new perspective in antibiotic therapy and the development of new antibacterial agents. Particular attention should be paid to the interdisciplinarity of the study conducted, which requires a Ph.D. Candidate's knowledge of different research fields and techniques.

I would ask for the Ph.D. Candidate to respond to the previously mentioned and following questions during public defense:

- Please explain more about the microfluidic devices used in the experiments. What were the dimensions of the microstructures in the microsystem for droplet formation, and why was such a chip geometry used? How does the microchip geometry influenced droplet size?
- Please explain what percentage of droplets were positive, *i.e.,* with docked cells, and whether it was possible to distinguish results for droplets with more than one cell? How was the repeatability of droplet formation?
- The droplet imaging chip was used to analyze the droplets. Could you explain why such geometry of the chip was used, and why you used the microchip instead of the standard plate?
- In the Discussion chapter, the Ph.D. candidate indicated *„ …. is that sub-MIC exposure to antibiotics induces ROS production, causing damage to cellular components such as DNA, membrane lipids…."* Please clarify whether any of the above parameters were verified within the doctoral dissertation. If not, is it possible to determine what changes in ROS can be expected in the performed study, depending on the type of antibiotic

used, for example? What other studies could be performed to confirm the mentioned hypothesis?

- One of the determined parameters was $(iMIC)_{exp}$. *However, the Ph.D Candidate didn't add any results, images based on which these parameters were determined*. $(iMIC)_{exp}$ is shown only in Fig RF2. Other Figures have $(iMIC)_{all}$. Could you comment that?
- The table caption should be placed above the table

Final conclusion

Taking into account the substantive value of the doctoral dissertation of M.Sc. Shakeel Ahmad, **I conclude that the evaluated doctoral dissertation meets the criteria for candidates applying for a doctoral degree as specified in the Act of July 20, 2018 Law on Higher Education and Science (Journal of Laws of 2023, item 742), in accordance with the provisions of the Act Art. 187. pt. 1., pt.2. and the evaluation of the submitted doctoral dissertation is positive.** What is worth emphasizing is the very good mastery and use of a wide range of modern research methods by the Ph.D. Candidate and the ability to develop them. Research related to the study of the influence of antibiotic pre-exposure below the MIC on heteroresistance at the single-cell level during subsequent antibiotic exposures to the same or different antibiotics brings important knowledge that can be applied in the future in biomedical research and diagnostics.The Ph.D. Candidate is co-author of 2 works under revision or preparation and co-authors of 6 other publications published in the journal from the JRC list. Ph.D. Candidate is also co-author of conference proceddings and seven conference presentations. **Due to the positive assessment of the entire doctoral dissertation presented above, I am applying to the Scientific Council of The Institute of Physical Chemistry (IChF) to admit** M.Sc. Shakeel Ahmad **to the next stages of the doctoral process, and based on the above, to distinguish this doctoral dissertation.**

Elżbieta Jastrzębska

Prof. D.Sc. Ph.D. Eng. Elzbieta Jastrzebska