Prof. WUT, Elzbieta Jastrzebska, D.Sc. Ph.D. Eng.
Faculty of Chemistry
Warsaw University of Technology
Noakowskiego 3, 00-664 Warsaw, Poland
ph.(+4822) 234 7253
elzbieta.jastrzebska@pw.edu.pl

Warsaw, 29 December 2023

Review of the doctoral dissertation
„Novel Microfluidic Strategies for Droplet Generation and Flow Control with Applications in Biotechnology"
of Ph.D. Candidate M.Sc. Francesco Nalin

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. Piotr Garstecki
Auxiliary supervisor: Dr. Marco Costantini

The doctoral dissertation presented to me covers broadly understood topics of microfluidic strategies for droplet generation and flow control with application in biotechnology. How was also underlined by Ph.D. Candidate, the development of microfluidic technologies in recent years has been rapid in fundamental research and application in various fields. At the same time, biotechnologies have been progressively evolving toward innovative directions and focusing on providing personalized medicine for patients. Microfluidic technology plays an increasing role in biological applications, therefore Ph.D. Candidate concentrated on this topic. Within this thesis, Ph.D. Candidate focused on developing and applying new microfluidic technologies, which can be useful in different biotechnology sectors. Ph.D. Candidate aimed to develop microfluidic-based solutions for current relevant biotechnological challenges.

The revised doctoral dissertation has six chapters: Introduction; Experimental with four Chapters, in which a short introduction, methods, and results have been included; Conclusions, and References (135 items). Additionally, the doctoral dissertation covers the Abstract and Contents. The entire doctoral dissertation (excluding Abstract and Contents) consists of 129 pages. The work was written in English and enriched with charts and numerous diagrams/drawings. On the editorial side, I would rate the work well. Some errors are noted at the end of the review.

The first part of the doctoral dissertation is Introduction. It covers 31 pages. The Introduction should introduce the thesis topic and show the newest achievement in the field to which the submitted work relates. In the first chapter of the Introduction, Ph.D. Candidate describes the basic information concerning microfluidics, such as laminar flow, surface tension, and wetting. The next chapter continues by describing methods applied in microfluidics. This includes microsystem fabrication methods such as soft lithography, surface tension mechanism (also in microfluidics), and surface modification methods. Subchapters 1.2.4 and 1.2.5 describe methods of droplet generation in microfluidics. Step emulsification is one of the methods used for droplet generation in microfluidic systems. Therefore, it is unclear why the author describes it as separate chapters. A solution to organize these chapters would be to create a subchapter/subchapters in 1.2.4. The last part of "Methods in Microfluidics" is the subchapter describing types of on-chip microvalves. I assume that Ph.D. Candidate intended to describe primarily the issues addressed in the dissertation. Nevertheless, I missed a broader view, at least signaling about other approaches used in the discussed field. For example, soft lithography is not the only method of microsystem fabrication but the only one cited by the author in this dissertation.

The last part of the Introduction describes three applications of microfluidics: cellular uptake of anticancer drugs, microfluidics for single-cell culture, and microfluidics for porous material. In this section, a deeper presentation of the current technological solutions in the field is missed to emphasize the essence of the microsystems fabricated by the Ph.D. Candidate.

The author writes about various approaches and examples without describing them briefly. For example:

Page 14 *"Therefore, **several solutions** have been reported, suggesting different surfactants for different microfluidic applications [14, 21–24]."*

Page 24 *"**Various methods** have been proposed for this purpose, with some similarities to the surface treatment techniques used in PDMS-glass bonding [57]."*

*Page 28 " For these reasons, **several strategies** for increasing the quantity of culturable bacteria from the HGM have been developed during the last few decades [79, 86–104].*

The literature presented in the thesis is new, mainly covering the last ten years. However, when the author would like to describe recent studies (Page 26, it is written *"Recent studies [66-69]*…), the citations should be younger than 2011 and 2014.

Without a doubt, I consider the Introduction to the subject of the work and the presentation of fundamental issues regarding microfluidics, as well as familiarization with the methodology that is reflected in the work, to be an advantage of this part of the doctoral dissertation. In particular when the doctoral dissertation has an interdisciplinary character. However, the author didn't present examples of current microfluidic systems applied in this research field. I think a short chapter summarizing the literature part of the work is also missing. At this point, I lacked

a clearly stated research hypothesis. Please clarify whether you established the hypothesis, and if yes, what the hypothesis of your doctoral dissertation is.

Experimental is the main part of the doctoral dissertation. It covers 79 pages, and is divided into four chapters (2÷5). The main goal of the thesis was to show that the proposed microfluidic systems are good solutions for current biotechnological challenges. Each subchapter of the experimental part contains a short introduction, materials and methods, results, and conclusions. However, the introductions mainly summarize what will be described in the chapters rather than outline the research problem to be solved or put forward a research hypothesis. Finally, at the end of the doctoral dissertation Conclusions are defined.

Chapter 2 describes the method (the combination of Time-Correlated Single-Photon Counting (TCSPC) with a microfluidic system) for the precise quantification of drug uptake time in cancer cells. The author concludes that the developed microfluidic device can be successfully used for precise drug delivery by integrating this device with a custom experimental setup coupled with (TCSPC). The recording the cellular uptake of anticancer drugs in cancerous cells can be done with sub-millisecond resolution. The main work of Ph.D. Candidate was focused on the technical part, which concerns the development of the setup, the microsystem, and the design of the software. In the results section, only the final results for cellular uptakes were presented without the measurement obtained for biological studies (although images of cultured cells in the microsystem). The final fabricated microsystem has not been also presented. I must point out that even though the proposed solution could have great potential in drug delivery, it is difficult to conclude this clearly based on the presented results in this thesis. This chapter mainly describes the methodology, in which the author did not avoid errors and omissions. The author does not provide information about the used cell line, how cell density was introduced in the microsystem, or what type of anticancer drug was analyzed. Concerning the technological part - please explain why the microsystem (scheme presented in Fig. 2.1) has serpentine inlet channels. If different solutions were introduced separately through each microchannel, it is not necessary to create a serpentine geometry, three straight microchannels are sufficient. Was it planned for a specific purpose? Why was the experiment with introducing a red ink in the microsystem presented for different microsystem geometry (Fig 2.7)? Please comment on why the proposed/fabricated microfluidic chip could be better for measuring drug uptake time in the cells compared with methods applied so far.

Chapter 3 discusses successfully isolating and cultivating bacterial species from the human gut microbiota based on microfluidic technology. This work was performed in cooperation with the Biomillenia SAS. The fecal and oral samples from 18 donors were collected and processed to extract the bacterial population. Ph.D. candidate developed the microfluidic system to encapsulate bacteria in monodisperse droplets from the bacterial suspension. The fabricated microsystem allowed high-throughput generation of droplets - 2-3 million within a few minutes. I assume that the size of the nozzles affects the size of the droplets obtained. The dimensions of the microstructures of the microchip are not given, only the height of the layers. How did the author select the dimensions of the microstructures, especially the nozzles, and how do their dimensions influence droplet size? The usage of microfluidic technology allowed the high-throughput encapsulation of single bacterial cells in droplets (under anaerobic conditions) and the creation of an extensive collection of bacterial strains

covering a diverse range of taxonomic units. There is no doubt that the author proved the potential application of microfluidic technology in comprehending the human microbiome.
Undoubtedly, Chapters 4 and 5 contribute essential knowledge in the field of new technologies for the use of microfluidic systems. Chapter 4 describes a new microfluid chip for the generation of droplets (both Oil-in-Water and Water-in-Oil) with a dynamic volume range. Microfluidic step emulsification was combined with on-chip microvalve technology. Thanks to that, the limitation connected with the requirement to fabricate a new chip for each size of generated droplet size. Real-time control of droplet size was made possible by modulating the pressure in the developed microsystem. What is more, high-throughput droplet generation was also received by creating a parallelization of nozzles in the microsystem. Finally, Chapter 5 describes the integration of the developed microfluidic system with 3D printing method. Thanks to that, the generation of functional hydrogels with different porosity and composition (by applying Y inlets in the microsystem) was possible. The developed technology will certainly impact and find potential applications in biomaterial engineering and biotechnology, for example cell culture. The author used three different biopolymer solutions (Dextran methacrylate (DexMA), Gelatin-methacryloyl (GelMA), and fibrinogen for for Oil-in-Water droplet generation. Only DexMA was selected for 3D Printing. What parameters determined the choice of this biopolymer for further research, and whether it was selected only based on the literature?
The last part is Conclusions. Throughout the dissertation, there is a lack of discussion of the results and discussion with the results of current research reported in the literature. Please comment the results obtained within this doctoral dissertation to the latest scientific literature.

Particular attention should be paid to the interdisciplinarity of the research conducted. Ph.D. Candidate carried out work on developing and producing microfluidic systems, culture, and analysis of cancer and bacterial cells, as well as fabricating new materials. This requires knowledge of different research fields and techniques. Ph.D. Candidate developed three different microsystems, which were also additionally modified. On-chip microvalve technology was implemented in the developed microsystems in such a way that high-throughput droplet generation was maintained. Undoubtedly, I would like to underline the research on the development of functionally hydrogels with different porosity and composition that deserves the greatest attention. This technological solution could be a milestone for further biological applications. Besides that, applying microfluidic technology for studying the human microbiome was also successfully confirmed.

I ask Ph.D. Candidate to address my comments above during public defense, especially the underlined ones. In addition to the above, I have added some additional comments and suggestions which could be helpful for future work of Ph.D. Candidate.

- Why are only the schemes of the developed microfluidic systems or fragments of microchannels shown in the thesis (for example, Fig. 2.1, 2.5, 3.5, 4.2)? There is a lack of photos of whole microsystems fabricated within this work. Some of the diagrams are difficult to read (microstructures of the microsystem are hardly visible in some schemes). Adding a photo of the fabricated microsystems would be useful.
- Several schemes concerning the technological part added in the thesis are without a particular description. It could be hard for researchers who are not similar to

microfluidics to understand what is on these schemes. (Fig. 2.2, Fig. 2.3, Fig. 3.2, , Fig. 3.5,).

- In Chapter 3. The scheme of the multilayer photolithography is shown, but the geometry of the chip is unclear.
- The thesis does not include the contents of the reagent, materials, and equipment. All needed information has been added to the text in Material and Methods. However, some data on reagents and equipment is omitted in this part.
- Page 11, line 3 – space is missing
- Page 39 *"20:1 elastomer-to-curing-agent ratio", "spinning the wafer for 45 seconds at 5500 rpm"* It is not clear how the parameters of PDMS preparation were chosen.
- Page 42 - It should be CO_2 instead of *CO2*
- Fig. 3.3., 3.4, 3.7 - axis descriptions are unreadable.
- Page 57 - *3 x 106 CFU/mL* it should be 3 x 10^6
- Page 77 - In the same paragraph, two different ways of writing *"Montecarlo*" and *"Monte Carlo"* are used.
- Page 78 - Other spacing is used.
- Page 85 - *" Figure 5.3 depicts the chip…"* Here is the mistake. It should be *Fig. 4.2.*
- Fig 3.5 and Fig. 4.3 - It should be μ instead of *u*
- Fig. 4.2 – The scheme of the microsystem should be described, for example, where the inlets are.

Final conclusion

Taking into account the substantive value of the doctoral dissertation of M.Sc. Francesco Nalin, **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 generation of functional hydrogels with different porosity and composition deserves special attention and emphasis. This technological solution could be important for further biological applications. Besides that, applying microfluidic technology for studying the human microbiome was also successfully confirmed. The Ph.D. Candidate is co-author of 3 works (1 related to the thesis) published in the journal from the JRC list. Another manuscript is under revision. He is also co-author of a patent and five international conferences. **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. Francesco Nalin to the next stages of the doctoral process.**

Elżbieta Jastrzębska

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