Dr hab. Piotr M. Korczyk
Zakład Biosystemów i Miękkiej Materii
Instytut Podstawowych Problemów Techniki PAN
ul. Pawińskiego 5B
02-106 Warszawa

Warszawa 03.01.2024

Review of the doctoral thesis by:

M.Sc. Francesco Nalin,

entitled:

Novel Microfluidic Strategies for Droplet Generation and Flow Control with Applications in Biotechnology

performed under the supervision of:

Prof. Piotr Garstecki (Supervisor)

and

Dr. Marco Constantini (Auxiliary supervisor)

at the Institute of Physical Chemistry of the Polish Academy of Sciences

The doctoral dissertation of Mr. Francesco Nalin, M.Sc. was submitted for review by the decision of the Scientific Council of the Institute of Physical Chemistry of the Polish Academy of Sciences.

The Ph.D. thesis focuses on the customization of microfluidic solutions, adapting current technologies or designing new ones for diverse applications in different sectors of biotechnology. The dissertation covers various projects, including the development of a method for the precise quantification of drug uptake time in cancer cells, the isolation and cultivation of bacterial representatives of interest from the human gut microbiota, the generation of droplets with a dynamic volume range, and the creation of functionally graded porous hydrogels with a high level of customization. Additionally, the integration of microfluidic technologies within a 3D printing platform for the manufacture of porous, functionally graded soft hydrogels relevant for the advancement of tissue engineering was also explored.

The dissertation is structured into six well-defined chapters, each highlighting a unique aspect of microfluidic technology and its application in biotechnology - from the development of methods for the quantification of drug uptake in cancer cells to the innovative creation of functionally graded porous hydrogels.

The main body of the dissertation is preceded by an abstract of the dissertation. It outlines the key objectives and motivations driving the research, highlighting the potential impact of microfluidic technologies in addressing challenges in healthcare, biotechnology, and material sciences. The author clearly states the main goals of the thesis.

The first chapter - the introduction of the thesis serves as a comprehensive overview of the advancements in microfluidics and its increasing relevance in various scientific and technological fields. It outlines the paradigm shift towards tailored therapies and personalized medicine, emphasizing the crucial role of microfluidics in this developing environment. The introduction also provides a glimpse into the specific applications of microfluidics that are explored within the thesis, indicating areas where microfluidics still has room for innovation and exploration. Overall, the introduction serves as a compelling prelude to the thesis, laying the foundation for the subsequent exploration of microfluidic technologies and their diverse applications in biotechnological challenges. The principles of microfluidics included at the beginning of the introduction are introduced in the thesis to provide a foundational understanding of the behavior of fluids at the microscale and the application of this knowledge. This part delves into the physics of fluids at the microscale, emphasizing the dominant forces, flow patterns, and transport properties that differ from those at macroscopic scales. It highlights the significance of viscous forces and molecular interactions over inertial forces at the microscale, leading to a range of events and practical applications that affect daily lives. The introduction demonstrates the author's deep understanding of the principles of microfluidics, including the behavior of fluids at the microscale, and his discernment in existing microfluidic biotechnology applications, which sets the robust stage for the subsequent exploration of novel methods and solutions within the thesis.

The following four chapters describe four different projects in which the author demonstrates the design, construction, and use of customized microfluidic platforms tailored to specific biotechnological applications.

The second chapter describes the first project that aimed in the development of a method for the precise quantification of drug uptake time in cancer cells. The author combines Time-Correlated

Single-Photon Counting with a microfluidic chip to control reagent flow, employing on-chip microvalves precisely. This chapter delves into the details of the experimental setup, the principles behind the quantification method, and the implications of the findings for understanding drug uptake dynamics in cancer cells. The Author provides insights into the experimental design, the microfluidic platform used, and the techniques employed to achieve precise quantification of drug uptake time. Overall, the second chapter offers a detailed exploration of the innovative methodology for studying drug uptake time in cancer cells, showcasing the author's valuable contributions to microfluidics and biotechnological applications.

The focus of the third chapter is on the isolation and cultivation of bacterial representatives of interest from the human gut microbiota. The author presents a microfluidics-based workflow with high-throughput encapsulation of single bacterial cells in droplets. The author describes the fabrication and the use of the high-throughput ‘microfluidic millipede device’, which creates 33 pl droplets of bacterial suspension in fluorinated oil. Droplets are then incubated and then distributed on microtiter plates to finally create a cell bank. Finally, the colonies were investigated by the use of Polymerase Chain Reaction to amplify the V4 region of the 16S rRNA gene for the final identification of the species in each well through sequencing. Additionally, the author created Monte Carlo simulations of bacterial species isolation to better understand the phenomena of culturing new species and make predictions on the anticipated outcomes from repeating the process. The approach described in the third chapter facilitates the creation of an extensive collection of bacterial strains spanning a diverse range of taxonomical units, including rare and potentially undiscovered species. That proves the significant advancements achieved through the development and application of the high-throughput microfluidic workflow, offering new possibilities for understanding and exploring the complexities of the human microbiome

The fourth chapter of the thesis focuses on developing Tuna-step - a novel microfluidic device for generating droplets with a dynamic volume. This chapter details the integration of microfluidic step emulsification with on-chip microvalve technology to achieve precise control over droplet generation and size regulation. The author proposed the device created by assembling two PDMS layers, providing a novel nozzle with an adjustable height due to the pressure actively applied to the deformable membrane. That allowed for tuning the size of generated droplets by setting the appropriate pressure, which was known from the performed calibration. Then, the author considered the parallelization of the nozzles for high-throughput droplet generation and both hydrophobic and hydrophilic surface modification for the generation of oil droplets in water and water droplets in oil,

respectively. The fourth chapter emphasizes the versatility and customization capabilities of the developed microfluidic device, particularly in creating functionally graded porous hydrogels with high customization. Furthermore, the author delves into the technical aspects and practical challenges related to the development of the microfluidic device, highlighting the successful resolution of intricate technical aspects through iterative optimization and innovative design.

The fifth chapter of the thesis focuses on the 3D printing of functionally graded materials using a microfluidic device. The section describes integrating the microfluidic Tunastep device with a custom 3D printer to generate porous materials that exhibit variations in both porosity and material composition. The section highlights the advantages of using microfluidic technology for 3D printing, including the ability to control the flow of materials precisely and generate complex geometries, showing the technology's potential applications in tissue engineering and other biomedical fields.

The last sixth chapter summarizes the main findings and conclusions of the study. It highlights the versatility and ease of customization of microfluidic technology for diverse applications in different biotechnology sectors. The section also emphasizes the successful resolution of practical challenges related to research's most intricate technical aspects. The author presents the outcomes of the doctoral thesis as concrete evidence of his dedication and contributions to the field of study.

The thesis ends with a list of 135 references.

Overall, the thesis is methodologically sound, with each original project underpinned by a robust experimental setup and logical progression of ideas. However, I found some points that could be improved I list them below:

- Incorrect structure of the description of projects. The author should structure the description more carefully, avoiding introducing terms or names before their definition or explanation. Such a situation appears, e.g., in chapter 2 on page 43, where the author refers to elements of his experimental setup such as FPGA, three-way valve switches, and so on, while those terms are described on the following pages. The final impression may suggest that some parts were shuttled just before the final editing of the thesis. That hampers the understanding of the project descriptions and requires secondary reading of the chapters.

- All claims and conclusions, mainly when any variable is quantified, should be supported by the explanation of specific data analysis and, if possible, with a graphical representation. Such a situation appears, e.g., on page 49, where the author states the value of quantified time to reach the proper drug concentration outside the cell. The reader knows only that this value was estimated based on the time-variation of the fluorescence signal. The description would be more complete if the author showed the plot of this signal and stated the methodology of estimating the threshold for the signal saturation.

- The same applies to estimating the operational conditions for the Tunastep device in section 4.3.2 on page 92. The author states that the maximum pressure applied to the membrane that ensures the generation of droplets was inversely related to the droplet flow rate. However, there is a lack of a graph confirming this statement.

- In the second chapter, the figures (Figure 2.1 and Figure 2.5) describing the microfluidic device for measuring uptake time in cancer cells are inconsistent. Although the same microfluidic chip is presented in both pictures, the names of the specific parts are not consequently repeated. For example, the names 'Outlets 1' and 'Outlet 2', marked in Figure 2.1, do not appear in Figure 2.5. Thus, the references to the parts of the experimental setup included in the main text are not unambiguous, which requires the reader's additional effort to understand the description.

- The author declares that one of his significant contributions to the presented works was the design of the microfluidic devices. Thus, the disappointing point of the thesis is that the microfluidic geometries are described rather briefly without providing geometrical details and without discussing the rationale behind choosing a specific solution. In the third chapter, the author mentions that he designed the chip based on the previously reported millipede device without describing the customization of this geometry to the specific research conducted within the thesis. The scheme of the millipede device (Figure 3.5a) is too brief and not supported with information about crucial dimensions. Similar concerns apply to the description of multiplied Tuna-step Nozzles in section 4.3.5.

Despite the comments mentioned above and concerns, I appreciate the high value of the dissertation presented by Mr. Nalin. Let me list a few vital points of the work:

- Interdisciplinary Approach. The thesis admirably integrates concepts from physics, engineering, biology, and materials science, reflecting a comprehensive understanding of the

interdisciplinary nature of microfluidics in addressing critical challenges in the field of biotechnology.

- Technical Mastery. Mr. Nalin exhibits exceptional skill in the development and application of microfluidic technologies. His work on on-chip valves and droplet generation techniques is particularly noteworthy.

- Innovative Solutions. The creation of the Tuna-step device for dynamic droplet generation exemplifies Mr. Nalin's ability to address complex challenges with innovative solutions.

- Practical Implications. The research has implications in drug discovery, microbiology, and biomaterial engineering, promising advancements in personalized medicine and biotechnological applications. The comprehensive exploration of microfluidic technologies and their applications in the thesis underscores the potential for transformative advances in personalized medicine, drug discovery, microbiology, and biomaterial engineering.

- Publication in Lab on a Chip. The research described in sections 3 and 4 was published in the Lab on a Chip journal by the Royal Society of Chemistry. The publication in such a renowned journal confirms the novelty of presented concepts, the high quality of the research, and the relevance of the challenged problems for society.

To summarize, the Ph.D. thesis titled "Advancements in Microfluidics for Biotechnological Applications" by Mr. Francesco Nalin, M.Sc., presents a comprehensive and innovative exploration of microfluidic technologies and their applications in various biotechnological challenges. These projects demonstrate the originality of the proposed solutions and their impact on relevant challenges in microfluidics, drug discovery, microbiology, and biomaterial engineering. Although the presented research was conducted in groups, the author clearly stated his own contribution, showing his unique and significant role. In my opinion, Mr. Nalin's contribution is more than enough to award him a Ph.D. degree.

The author's research demonstrates high technical proficiency and originality in developing and integrating microfluidic technologies for diverse applications in different biotechnology sectors. The thesis encompasses four significant projects, each addressing relevant challenges in microfluidics, drug discovery, microbiology, and biomaterial engineering. The author's contributions include the development of novel methodologies for precise drug uptake time

measurement in cancer cells, the isolation and cultivation of previously uncultured bacteria from the human gut microbiota, the creation of a novel microfluidic device for generating droplets with dynamic volume control, and the generation of functionally graded porous hydrogels using microfluidic devices in combination with a custom 3D printer.

Overall, the thesis presents concrete evidence of the author's dedication and significant contributions to the field of study. The research outcomes underscore the potential of microfluidic technologies in addressing critical challenges in biotechnology and healthcare, making it a valuable and impactful contribution to the scientific community.

Recommendation

Considering the above, I'm pleased to conclude that Mr. Nalin's doctoral thesis meets the conditions set out in Art. 187 of the Act of July 20, 2018, Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2023 r., poz. 742 ze zm.).

Due to the above-mentioned positive assessment of the entire doctoral thesis, I recommend the Scientific Council of the Institute of Physical Chemistry of the Polish Academy of Sciences to admit Mr. Francesco Nalin, M.Sc., to further stages of the procedure for awarding the doctoral degree.