Uniwersytet Wrocławski

WYDZIAŁ BIOTECHNOLOGII

ZAKŁAD CHEMII BIOLOGICZNEJ
ul. Joliot-Curie 14a, pok. 3.12
50-383 Wrocław

tel. +48 71 375 27 65

www.chembiolab.uni.wroc.pl

Professor Artur Krężel
Department of Chemical Biology
Faculty of Biotechnology
University of Wroclaw
Joliot-Curie 14a
50-383 Wrocław

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Review of the doctoral dissertation by Francesca Canyelles i Font M.Sc.
entitled „Development of dual-analyte fluorescent probes for the detection of hypoxia in cellular models"

For at least two decades, the development of analytical chemistry has been heavily focused on the design of fluorescent probes for precise measurements of analyte concentrations in cellular environments, or parameters related to the functioning of the cell and its compartments, such as redox potential, polarity, pH, metal ion concentration, etc. Currently, increasingly advanced probes are being developed, allowing for the simultaneous monitoring of several parameters. Similarly, multifunctional probes are being developed, which, on one hand, allow for the monitoring of desired concentrations or parameters, and on the other, can be specifically localized within the desired structure. The reviewed doctoral dissertation by Francesca Canyelles i Font M.Sc. fits precisely into this line of research. The work was carried out at the Department of Molecular Probes and Prodrugs of the Institute of Bioorganic Chemistry of the Polish Academy of Sciences under the supervision of Dr. habil. Jacek Kolanowski. The doctoral dissertation by Francesca Canyelles i Font M.Sc. is an example of well-performed project at the intersection of organic and analytical chemistry, biological chemistry, biophysics, and cell biology. It represents a continuation of the long-term research conducted by the supervisor, Dr. habil. Jacek Kolanowski.

The doctoral dissertation submitted for my review, consisting of 264 pages, follows the format of a traditional doctoral thesis and is written in English. It includes a theoretical introduction, aims of the work, results combined with a discussion, conclusions and outlook, a methodology section, an appendix, and a bibliography containing 180 references. At the beginning of the dissertation, there is a list of abbreviations used, which is extremely useful when reading the text due to the large number of them. It is worth noting that the appendix is very extensive, accounting for one-third of the dissertation. It contains NMR spectra (proton and carbon) and HRMS spectra, demonstrating the identity and purity of the obtained chemical compounds. Additionally, it includes supplementary spectra (including entire experiments) of absorption and emission, tables showing cell viability tests, determination of IC_{50} parameters, and flow cytometry scatter plots.

The 25-page introduction is a concise chapter containing all the necessary information for understanding the topics discussed in the paper, the need for research, as well as the analysis and discussion of the results. In this chapter, the author presents the physical principles of fluorescence and the optical parameters characterizing compounds exhibiting such properties. The mechanisms by which fluorescence quenching occurs, which are crucial for designing off-on type sensors, are also addressed. The basics of sensor design and construction are described, with particular emphasis on the fluorophores themselves, including hemicyanine dyes and coumarin. The chapter dedicated to dual-analyte probes, which is very important given the subject of the work, is rather brief without a detailed breakdown of the types of such probes. It presents one type of sensor sensitive to Fe(II) ions and hydrogen peroxide. The last part of the introduction discusses hypoxia as a phenomenon and characterizes the microenvironment in which it occurs. The Author rightly emphasizes that most constructed probes are single-analyte probes for sensing hypoxic states. She points out that, to date, only one dual-analyte probe has been developed for hypoxia and ATP, which reports the changes under conditions of disrupted metabolism. The Author thus highlights the need for the construction of multi-analyte probes for the detection of hypoxia and related analytes. She emphasizes that the pH changes occurring in the microenvironment of cancer cells (acidosis) could be utilized in the construction of such probes. With this conclusion, the Candidate smoothly transitions to the aims and objectives of the performed experimental work, which are divided into three parts: (i) design of dual-analyte probes for hypoxia, including the selection of appropriate fluorescent scaffolds and the selection and positioning of pH- and redox-sensitive residues; (ii) synthesis and characterization of the obtained probe; and (iii) validation of the probe for hypoxia in cellular models.

From the description of the results, it is evident that the Candidate chose a hemicyanine core with a pH-sensitive moiety as the focus of her work, which can act as an anchoring site for a second sensing analyte and as an anchoring point for targeting or ratiometric purposes. In the first part of the work, the Author presents a clear and understandable description of the design and synthesis of hemicyanine dye derivatives (HCy-OMe, HCy-NEt_2, and HCy-Jul). She clearly indicates which of the obtained compounds had been previously synthesized and which was obtained for the first time in this work (HCy-Jul). She then proceeded to characterize the fluorescence properties of the dyes. These properties were studied in DMSO (why, since they are intended for biological applications and are supposed to function in aqueous solutions?), and the results, as expected, show low quantum yields of the fluorophores in this form, particularly for HCy-OMe. Concentration studies did not reveal any issues with aggregation and showed that the compounds can be used in micromolar concentrations. In their current form, the compounds do not change their fluorescence in response to pH. However, when they were tested in different buffers—phosphate, Tris-HCl, and HEPES at the same pH—their fluorescence varied, especially in the latter buffer, suggesting interactions between the buffer components and the fluorophore. All dyes show an enhancement

in fluorescence with increasing viscosity to a similar extent. The fluorescence response to changing polarity is also similar, although there are some differences between the substituents in the position 3. All the compounds tested exhibited stable fluorescence in the presence of a range of inorganic salts (stability towards s- and d-metal cations). I have a comment regarding the salt $[Cu(MeCN)_4]BF_4$. The Cu(I) cation is stabilized in aqueous media only above 10% volume concentration of acetonitrile; otherwise, it undergoes disproportionation. This means that, in fact, the effect of Cu(I) ions may not have been examined under the applied conditions. The Author also tested the fluorescence properties of the compounds in cellular media (regular DMEM High Glucose and FluoroBriteTM) to assess their suitability for cellular purposes. Among all the derivatives tested, HCy-Jul was characterized by the greatest fluorescence stability. Interestingly, this stability was different in typical cell media. Why is fluorescence more stable in DMEM medium containing albumin than in FluoroBrite medium? What is the reason for this?

An interesting stage of the research was determining the stability of the dye derivatives against selected nucleophiles such as cyanide (CN^-) and methanethiolate (CH_3S^-), using NMR to examine 1,2-addition and 1,4-addition, respectively (unbuffered system, 80% DMSO and 20% D_2O). In the case of the first nucleophile, all dyes underwent addition, while for the second one, only HCy-OMe did. The stability of the dyes was also tested against the cellular reductant, reduced GSH. In this case, only HCy-Jul remained unaffected. However, the exact reaction that occurred for the other two compounds is unclear to the Author. Would the use of LC-MS or LC-MS/MS not shed more light on the nature of the reaction? Continuing the investigation into the effect of nucleophiles, the Author decided to check whether the HEPES buffer component could also play such a role. Tests showed that increasing the pH to 9 had almost no effect on HCy-Jul and HCy-NEt_2, but caused a significant drop in fluorescence for HCy-OMe. The addition of GSH to HEPES further increased the differences between the hemicyanine derivatives and confirmed the nucleophilic character of HEPES and the nucleophilic resistance of HCy-Jul. Why were the above studies and the NMR analysis conducted in such a high concentration of DMSO? The spectral characterization of the compounds was not performed at such high DMSO concentrations.

In the final stage of the first part of the research, the Author described the results obtained using two cell lines, HEK239 and the cancer line DU145. These showed that all the tested dyes can pass through the cell membrane and localize inside the tested cells. Importantly, co-localization studies with organelle-specific stains (MitoTracker and LysoTracker) showed that HCy-NEt_2 and HCy-Jul localize partly in the mitochondria and cytoplasm but do not localize in lysosomes. This is very significant for their potential use in the construction of hypoxia sensors. The naturally low pH in these organelles would affect the fluorescence readout and interfere with the response to hypoxia. Viability studies showed that both compounds, tested at this stage, can be used in micromolar concentrations.

The conducted studies showed that HCy-Jul best meets the requirements for fluorescent sensors and is also the most stable among those tested. For this reason, it was chosen in the next part of the research to construct the actual hypoxia sensor, NHCy. The synthesized sensor was characterized in terms of its optical properties. As expected, it did not show emission changes in response to pH. However, the reduction product of the sensor (compound 5, unnamed unfortunately) does exhibit pH sensitivity. The presence of an -OH group instead of $-NO_2$ eliminates fluorescence quenching, making this compound highly bright. Its emission properties depend on pH (it emits at low pH), automatically making it a dual sensor for hypoxia and pH. Emission measurements in different buffers at varying pH values (HEPES was not used, rightly so) showed that the compound's pK_a is 5.8, which fits well for its potential use as a sensor for relatively low pH environments. Figure 46b shows that 12 measurements were used for characterization, while Figure 47, which displays the data fitted to the logarithmic version of the Henderson's equation, only shows seven points. Why is that? Why did the Author not use, for example, the Hill equation, which not only allows for the determination of the pK_a value but also the cooperativity coefficient? To demonstrate the full functionality of the NHCy sensor, the Candidate used nitroreductase (NTR) with NADH for its reduction *in vitro*. NTR converts the nitro group to a hydroxyl group, increasing fluorescence of the probe, provided the pH is sufficiently low. What is the rate of this reaction under the conditions used? I assume that the rate is high, as is typical for most enzymes.

In the next step, studies were conducted on two cancer cell lines, A549 and DU145. It is unfortunate that there was no control using non-cancerous cells, where hypoxia occurs at much smaller extent. The viability tests showed that both cell lines can be used when the sensor is applied at a concentration of ~2 μM. Next, fluorescence confocal microscopy of the cell lines treated with NHCy in normoxia and hypoxia (induced for 4h at 1% O_2) was performed. The experiments with the DU145 line demonstrated the full functionality of the sensor, showing increased fluorescence under oxygen exposure. Additionally, co-localization studies with trackers showed no localization in lysosomes, meaning there was no interference from the low pH in these organelles on the pH decrease under hypoxia. In the case of the A549 line, the experiments were inconclusive, which is why the Candidate, together with collaborators, conducted 3D studies (tumor spheroids) on this line. Despite several interesting observations, the conclusions were also unclear, indicating that the sensor cannot be used in this line. The Author attributes the differences between the cell lines, among other factors, to their different redox buffering capacities. Following this line of thinking, the cause could also be due to differences in pH buffering capacity, as both factors influence the fluorescence properties.

The final stage of the research involved the construction of a ratiometric dual-analyte probe for hypoxia. The use of an additional fluorophore increases the resolution of fluorescence measurements and allows for much better calibration of the sensor in cellular conditions, making it independent of

concentration, among other advantages. These were the benefits the Author aimed for in designing the NHCy-C probe, which contains a coumarin scaffold. After synthesis and purification, the compound was spectrally characterized; however, in my opinion, not as thoroughly as the earlier characterization of the dyes themselves. There is a lack of detailed pH-metric studies (no titrations or determination of pK_a values) for both the sensor and the product of the NTR enzymatic reaction. This compound responds to both hypoxia and low pH. Importantly, the sensor was processed by the NTR enzyme in the presence of NADH, which is visible as a band appearing around 625 nm when excited at 420 nm. The coumarin band remains unchanged. The Author also did not examine the effect of metal ions on fluorescence stability. The expanded structure of NHCy-C increases the probability of interactions with metal ions. The sensor was applied to three cancer cell lines (without a non-cancer control line). However, what was done is the measurement of the sensor's stability in the presence of GSH within a pH range of 7-9, showing its resistance. After testing cell viability, the probe was used at a concentration of 2 μM. Co-localization studies showed no presence in lysosomes but presence in mitochondria and cytosol, in slightly different ratios depending on the cell line used. The sensor's functionality was tested using both confocal microscopy imaging and flow cytometry. The Author demonstrated that the latter method produced more resolved results, indicating correct dual activity of the probe in response to both hypoxia and reduced pH. Can the Candidate explain the reason for this better resolution? What is missing in the final part of the work is a discussion of the calibration potential of the sensor response offered by the ratiometric system, as well as an analysis of the results through the measurement of green and red fluorescence of both bands.

The dissertation lacks a separate discussion of results chapter. However, this does not mean that there is no discussion in the work. It has been integrated into the description of the results, and each experimental chapter concludes with a brief summary. This approach is very convenient as it allows the reader to summarize the findings before reaching the end of the dissertation. These chapters are well-written and include summaries of the most important observations or conclusions. However, the thesis missed a deeper discussion of the results in comparison with literature data. The Author cited a considerable amount of specialized literature in the introduction, which could have been used in the discussion of the results. Nevertheless, the conclusions drawn were correct. The dissertation concludes with a one-and-a-half-page final summary, which includes a vision for further work on hypoxia probes. The methodological section was well-prepared and very clear. However, there is no information about the origin of the NTR enzyme and NADH.

The dissertation submitted for review presents a very well-designed doctoral project. The work is written in correct language, and I found only minor linguistic errors. Among the errors resulting from the use of English, punctuation mistakes and the absence or unnecessary use of articles should be mentioned. The dissertation is accompanied by clear and well-prepared illustrations, though Figure 26 is of low

resolution. On page 44, there is a very visible, bold error: "Error! Reference source not found." It is unclear which reference the author is referring to.

I have no major substantive objections to the reviewed work. However, in addition to the questions I raised out of curiosity, I would like to add the following:

- The substrate of the enzymatic reaction involving NTR, which in this case is the NHCy-C sensor, is weakly fluorescent. The product of the enzymatic reaction at neutral pH (far from acidic) is also weakly fluorescent. Is there a way to distinguish between the neutral pH state and the possibility that the reaction has not occurred at all?
- Referring to the previous question, would the use of a probe dedicated to measuring pH over a wide range help in distinguishing this state? If so, why didn't you determine the pH in the cells being studied? The use of non-cancerous cell lines could also serve as a useful control.
- While reading the dissertation, I had the impression that the Candidate devoted a great deal of work to the characterization of dye derivatives. Fewer such studies were conducted on the final version of the sensor. Please provide a comment on this?

In summary, despite certain shortcomings mentioned above, I find that I have the opportunity to review a very interesting work whose results are valuable both technically and scientifically. The thesis by Francesca Canyelles i Font M.Sc. is an example of a well-executed interdisciplinary research project under the guidance of an experienced supervisor. Therefore, I conclude that the doctoral dissertation submitted for review by Canyelles i Font fully meets the conditions set forth in the Law on Higher Education and Science of July 20, 2018 (Journal of Laws of 2018, item 1668, as amended) of July 3, 2018, introducing provisions of the Law on Higher Education and Science (Journal of Laws of 2018, item 1669, as amended) as well as the procedures for conferring the doctoral degree at the Institute of Bioorganic Chemistry of the Polish Academy of Sciences in Poznań (Resolution No. 28/2024/Internet of the Scientific Council dated March 20, 2024). I therefore recommend to the Scientific Council of the Institute of Bioorganic Chemistry PAS that Francesca Canyelles i Font be admitted to the further stages of the doctoral degree conferral process.

Respectfully yours

Artur Krężel