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

The doctoral thesis of Ms. Francesca Canyelles i Font, M.Sc. was prepared in the Department of Molecular Probes and Prodrugs in the Laboratory of Molecular Assays and Imaging of the Institute of Bioorganic Chemistry of the Polish Academy of Sciences under the supervision of Dr. Hab. Jacek Ł. Kolanowski.

Optical imaging is one of the most dynamically expanding imaging techniques. Rapid technological advances in the last few years have enabled the use of optical imaging strategies among other methods to monitor hypoxia levels. Great advantages of this technique include its real-time feedback, resolution down to the nanometer scale, and safety (lack of ionizing radiation) that allows repeated measurements in the course of the treatment, with the additional advantage of inexpensive and mobile equipment. The use of optical imaging is only limited by the penetration of light in tissue (mm– cm), making it applicable only for solid tumors located close enough to the surface of the skin or close to the optical fiber placed inside the body. However, it is a very promising technique for intraoperative guidance, where tumors can be directly visualized. The application of appropriate probes might extend its application to the evaluation of hypoxia state in a solid tumor after biopsy. Therefore, there is a great challenge and high demand in the development of fluorescent probes, which are selective toward hypoxia tissue. The studies undertaken by Ms. Francesca Canyelles i Font are part of this research subject and are a continuation of the research conducted in the team of Dr. Hab. Jacek Kolanowski.

The doctoral dissertation submitted to me for evaluation begins with acknowledgments and an indication of the projects within funding that the research was conducted for this dissertation. It continues with a table of contents and abstracts in English and Polish, followed by a list of abbreviations. The first chapter of the dissertation is the theoretical introduction, covering 25 pages. It is very well prepared and provides an excellent introduction to the issues covered in the dissertation. The chapter begins with basic concepts related to the phenomenon of fluorescence, the identification of fluorophores in biological systems, and the basics of fluorophore-based cell imaging. Next, Ms. Canyelles i Font describes the idea of designing the responsive probes: i) fluorophores focusing on hemicyanine and coumarin dyes, ii) the difference between reversible and irreversible probes, and iii) dual-analyte probes. The last paragraph is devoted to the basic information on the redox balance in cells, the occurrence of hypoxia, and acidosis in the tumor environment. There is only a small paragraph on fluorescent

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probes for hypoxia, which in my opinion is not enough. There are plenty of such systems and, in particular, information on their weaknesses would be valuable, which would justify the need to undertake research toward obtaining better systems.

In the next chapter of the work, the Ph.D. Student presents the purpose and plan of her research. The main objective of the reviewed work was to develop hypoxia probes that are based on dual-analyte fluorescent molecules. The designed molecules should be able to monitor at least two analytes associated with hypoxia. To achieve this goal, the Ph.D. Student used data available in the literature and planned her research which comprise the following items:

1. Finding an appropriate fluorescent scaffold which will be stable in the biological environment and will allow the possibility of modification toward the introduction of responsive moieties toward pH and nitroreductases;
2. Synthesis and characterization of the dual-analyte fluorescence probe;
3. Validation of the probe in the in vitro and cellulo environment.

The following work includes the *Results and Discussion* chapter, which is divided into four subchapters. In the first subchapter (3.1) covering two pages, the Ph.D. Student discusses her approach to design the dual-analyte fluorescence probe based on the literature data.

The next subchapter (3.2) is devoted to three hemicyanine derivatives that have various electron-donating groups (EDG) in the aromatic ring of the fluorophore, namely methoxy (HCy-OMe) or diethylenoamine (HCy-NEt2) in the para position or forming the Julolidine substituent in the meta position (HCy-Jul).).). The first two compounds have been previously reported and the last is the new scaffold. It must be emphasized that all three compounds were successfully synthesized and fully characterized by NMR and mass spectrometry (HR-MS). To discuss the electron-donating/electron withdrawing (EWG) character of aryl substituents for synthesized compounds Ms. Canyelles i Font used of Hammett parameters. Spectroscopic properties such as absorption coefficient and quantum yield of fluorescence were measured for HCy-NEt2 and HCy-Jul in DMSO. The quantum yield of the fluorescence of both compounds was very low. Furthermore, the impact of pH, type of buffer, viscosity, and polarity on the fluorescent properties of the compounds studied was measured and extensively discussed. An important experiment was to check the stability of the probes in cellular medium, and of all probes tested, HCy-Jul was the least degraded/quenched. To find the reason for the decrease in fluorescence intensity, the impact of various metal ions that might be present in cellular medium was checked and was found to have a marginal effect. Furthermore, the probes were exposed to nucleophiles such as cyanide and methanethiolate and high stability was found for HCy-Jul and moderate for HCy-NEt_2. Based on the experiment performed, these two probes were further studied in vitro in two cell lines DU145 (human prostate cancer) and HEK239 (immortalized human embryonic kidney cells). It was not possible to measure IC50 due to concentration issue; however, the dose used for staining cells was low enough to be found nontoxic. The imaging data obtained from the confocal microscope showed that the probes accumulate in the cytoplasm and mitochondria; however, the difference between the autofluorescence of cells and that of probes treated with them is alarmingly low. Usually, a one-order-of-magnitude

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difference is considered suitable to use the probe for bioassays. Taking into account all of the experiments performed, a Ph.D. student found the HCy-Jul probe good enough to perform further modification towards obtaining the dual-analyte fluorescence probe.

This part of the dissertation raises the following questions:

1. As the compound HCy-NEt2 has previously been reported, are there dates for its absorption coefficient and quantum yield of fluorescence in the literature that can be compared to those obtained in this work? What about compound HCy-OMe have you measured these parameters for this one?
2. One of the serious problems of fluorescent probes is photobleaching; have you tested your probes toward evaluation the photoinduced decomposition of probes?
3. The quantum yields of fluorescence and brightness are very low; could you find in the literature these values for commercially used dyes based on the hemicyanine scaffold? Compounds are obtained as salts with iodine as a counterion, which is known as a quencher. Have you considered the presence of these ions on fluorescence intensity?
4. The quantum yield of fluorescence was measured in DMSO, while many of the experiments were performed in an aqueous solution; therefore, it would be good to know the quantum yield of fluorescence also in water or buffer. Or at least to run the emission spectra under both conditions, that would also give some relative information about emission properties. Most of the dates given in the dissertation are presented in the normalized scale.

The following subchapter (3.3) is focused on modification of the HCy-Jul probe to obtain probes that respond to bioreduction performed in the presence of NTR-like activity. Additionally, the reduced species should undergo a transformation that results in a probe responding to changes in pH. The creation of such a probe is a big challenge. The theoretical considerations for the design of such a probe have been carefully planned on the basis of reports from the literature. Synthesis of the plan compounds was successfully completed, resulting in two probes for further studies of NHCy (possessing a nitrophenol substituent responsive to NTR) and compound 5 being a postulated product of NHCy reduction catalyzed by NTR in the presence of NADH. The latter compound was named the bio-reduced form of NHCy. In Schemes 3 and 4 the same numbers are given to different compounds that might be confusing. The spectroscopic properties such as the absorption coefficient and quantum yield of fluorescence were measured for NHCy, and again the fluorescence quantum yield was very low. The probe was resistant to glutathione, so it could not be nonspecifically reduced by this reductant. It must be noted that the emission for NHCy probe was pH independent, while the emission for the bio-reduced form increases with lowering pH; the estimated pKa value was 5.8. The reduction of NHCy by NADH was demonstrated in the presence of NTR, which confirms the proposed reduction scheme. *In vitro studies showed that this compound has an IC50 ca.* 1 μM against DU145, while in the case of A549 cells (adenocarcinomic human alveolar basal epithelial cells), its cytotoxicity was much lower, reaching 20% cell death for 10 μM. Generally, to determine the cytotoxicity of a new compound, it would be necessary to compare it with a known cytotoxic compound; for this purpose, cisplatin is often used. The

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staining properties of NHCy were evaluated on two cell lines DU145 and A549, in both cases the difference between autofluorescence and cells treated with NHCy was approximately twice, only in the case of hypoxia in A549 cells the difference reached approximately 3-4 times higher fluorescence intensity values. The imaging properties of NHCy were also evaluated using spheroids formed from DU145 cells. The difference in fluorescence intensity for treated and untreated cells was rather small.

This part of the dissertation raises the following questions:

1. I would recommend to check the time-dependent changes in fluorescence upon the reduction of NHCy by NADH catalyzed by NTR.
2. Looking at Figure 46 it can be concluded that at the pH ca. 7.4 bio-reduction of the probe under hypoxic conditions should lead to a decrease in fluorescence emission (if a full reduction occurs), while in more acidic conditions at pH 6-6.5 the difference between both forms is minimal. This trend in DU145 cells is preserved, while in A549 cells is reversed (Figure 52) could you explain this.
3. Hypoxia in the cell was induced by keeping them for 4 h in the cell incubator chamber. Could you describe what kind of incubator chamber was used, how 1% O2 was reached and if the medium was also prepared in advance at the same atmosphere?
4. Have you checked necrosis in the formed spheroids, e.g. using propidium iodine staining?

The next subchapter (3.4) starts from the sentence: *The probe described in the previous chapter exhibited a largely turn-on-type response to hypoxia, i.e. the intensity of fluorescence increased in hypoxic conditions.* In my opinion, the word 'largely' is not appropriate, and also the origin of the response in cells is not clear. This section is devoted to the synthesis of the new dye by linking the coumarin fluorophore to the NHCy. The new probe labeled NHCy-C was successfully obtained. The spectroscopic data for these ratiometric probes are the lack of absorption coefficient or quantum yield of fluorescence. Only absorption and emission spectra recorded in PBS pH 7.4 are given. The reaction with NADH catalyzed by NTR revealed that the probe responds to the enzyme, and the distinct ratiometric signal differentiation of the untreated from the treated with the NTR probe can only be obtained for pH 4. In vitro studies were performed for three cell lines DU145, A549 and MCF7 (breast cancer) cell lines. The highest cytotoxicity was observed for DU145 cells, while for two other lines it was non-toxic in the tested concentration range. Confocal microscopy studies showed that for DU145 cells i) autofluorescence is pretty high compared to the signal from a probe: ii) there is a low difference in emission under hypoxia (approximately 10%); iii) coumarin fluorophore is not sensitive to normoxia/hypoxia conditions, which is beneficial for ratiometric probes. The studies on MCF7 cells were carried out in collaboration with two institutes in Spain. The colocalization of this probe was similar to that for previously investigated NHCy, the cytoplasm, and the mitochondria. Evaluation of staining properties was done using flow cytometry which allows one to minimize the autofluorescence effect at channel 3 (ext/em 405/610) and this allows one to obtain much higher difference in emission between cells cultured under normoxic and

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hypoxic conditions. To prepare cells under hypoxic conditions, a special hypoxia chamber was used. For A549 cells, no staining data were reported.

This part of the dissertation raises the following questions:

1. The caption for Figure 61 is not clear; could you explain better what is in this figure?
2. In summary, it was written: *'It would be the first reported dual-analyte ratiometric probe for hypoxia by surrogate sensing of two characteristic parameters of such state (NTR activity and pH)*' Could you explain how you check the ability to sense pH in cells by NHCy-C?
3. One of the fundamental questions about the safety of probes is their photocytotoxicicity. Have you considered experiments that would check the photocytotoxicity of newly synthesized dyes?

The chapter *Results and Discussion* is written in a clear way, with numerous figures and tables. However, in some of the figures, the axis captions and legends are difficult to read. It should be emphasized that the Ph.D. Student was provided with a huge amount of data, and all the obtained research results are very meticulously discussed in relation to existing literature reports and critically analyzed.

In the next chapter of the work, the Ms. Canyelles i Font has included a summary, which briefly presents the most important conclusions drawn from the conducted research and comments on the received data in terms of the broader perspective of the possibility of their use for further research. In my opinion, the prepared summary is too general. Undoubtedly, the most important achievement of the Ph.D. student was the obtaining of new probes by advanced synthesis. Demonstration that ratiomateric probes show emission at one wavelength invariant because of pH changes and the presence of nitroreductases, while at other wavelengths such a dependence is observed, was successful. Monitoring hypoxia, however, is still unclear, and the main obstacle is the pH dependence of fluorescence emission. In cells even under hypoxia, pH as low as 4 is not observed, for which there are the greatest changes in fluorescence intensity, while for higher pH the changes due to bio-reduction are negligible. It seems that this aspect should be taken into account in further work on this probe.

The next chapter of the work, 'Methodology', is well prepared. The synthesis of each compound is described in detail, and, accompanied by the corresponding NMR and MS analysis, the compounds were obtained with decent yields, which is undoubtedly a great success for the Ph.D. Student. In addition, there are descriptions for all the experiments performed. The descriptions presented are very reliable, indicate a very good knowledge of the doctoral student's research technique, and indicate the immense amount of work that she did both during the syntheses and the analysis of the properties in model systems as well as *in vitro*.

An Appendix containing, among others, NMR spectra and additional data in the form of graphs and tables from the experiments carried out, to which the doctoral student refers in the main text of the dissertation, is included. At the end, there is a bibliography of 180 items.

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In summary, the data obtained, their analysis, and interpretation are of high level. In this work, a very extensive research material was presented, demonstrating the excellent preparation of the Ph.D. Student to conduct syntheses of complex systems and to conduct photophysical and in vitro studies using confocal microscopy. This type of research required good preparation both in the synthesizing of compounds, the preparation of samples, the performing of experiments, and the analysis of the obtained data. In all these aspects, the doctoral student demonstrated great professionalism and scientific maturity. In summary, my evaluation of Ms. Francesca Canyelles i Font's doctoral dissertation, I would like to emphasize the high substantive level of the research carried out and hope that it will be published soon.

The doctoral dissertation submitted for review meets the conditions specified in the Law of 20 July 2018 - Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended), and the Law of 3 July 2018. Introductory Provisions of the Law - Law on Higher Education and Science (Journal of Laws of 2018, item 1669, as amended), and in the Procedure for the Award of a Doctoral Degree at the Institute of Bioorganic Chemistry PAS in Poznań (Resolution of the Scientific Council of ICHB PAN No. 28/2024/Internet of March 20, 2024), and I request the Scientific Council of the Institute of Bioorganic Chemistry PAS to admit Ms. Francesca Canyelles i Font to further stages of the proceedings for the award of a doctoral degree.

Podpisany elektronicznie przez
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