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Referee report

On the doctoral thesis by MIROSLAV KUBA, entitled

Novel fluorescent nucleotides for metabolic labelling and for the construction of DNA probes

In Prague, September 5th, 2022

The dissertation thesis by Miroslav Kuba summarises the synthesis of novel fluorescently labelled nucleosides and nucleotides and their enzymatic incorporation into DNA by primer extension (PEX) and polymerase chain reaction (PCR). The labelled (oligo)nucleotides were used for sensing interactions with proteins, FLIM microscopy, real-time imaging of DNA synthesis in live cells and super-resolution imaging of DNA. As demonstrated by the broad scope of applications, the development of new fluorescently labelled oligonucleotides is a research topic of high scientific relevance.
Parts of the thesis seem to be published as two first-author publications in *Tetrahedron* and *Chem. Eur. J.*, but the text does not specify which parts were already published. The candidate further co-authors two more manuscripts published in 2021. The thesis reveals that the candidate has a broad skillset ranging from synthetic organic chemistry of various fluorescent dyes and nucleosides and the corresponding triphosphates, chemical biological methods for enzymatic nucleotide incorporation into oligonucleotides (ONs) and DNA, characterisation of prepared ONs, and spectroscopy of fluorescently labelled compounds. The thesis is of experimental character, focusing mainly on the characterisation and application of prepared ONs.
The dissertation begins with a theoretical introduction to DNA, its synthesis and functionalisation, and fluorescent dyes. The Results and Discussion part of the thesis contains five parts (six chapters), discussing the preparation and testing of ONs labelled with various fluorescent dyes (tryptophan-based imidazolinone, benzylidene-tetrahydroxanthylium, thiazole orange, dimethoxy-, diphenyl-BODIPY, and silicon rhodamine). Division of the text based on various fluorophores seems to be a good idea, but as the methodology was very similar for each fluorescent label (synthesis, spectroscopy, PEX, PCR, applications), the dissertation tends to be highly repetitive. What more, any comparison between differently labelled ONs is completely missing. This brings several conceptual questions to the referee's mind:

1) What was the general aim of the work? What is the ultimate target? Why were so many structurally and spectrally different fluorescent labels selected as specific aims? Were all the specific aims formulated at the very beginning of the thesis to have a pool of variously fluorescently labelled ONs, or were some alternative targets added when certain dyes did not perform as initially expected (e.g., as a second generation)?
2) If the candidate were to pick one of the fluorescently labelled ON, which one would it be and why?

Generally, the text could have gone through more detailed proofreading as it contains numerous typographical and factual errors. Here, I attach a list of some findings:

List of abbreviations: pm is used for parts per million; the convention uses ppm
P 16: phosphordiester vs phosphodiester

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P 16: helixes vs helices
P 27: Scheme 8: POCl3 vs $POCl_3$
P 30: CuAAC reaction produces 1,4-disubstituted 1,2,3-triazoles, not 1,5-disubstituted
P 45: sensitives vs sensitive
P 47: acrylamide **33** vs propargylamide **33**
P 49: To compare the relative emissivities in Figure 13D, one needs to excite in the isosbestic point, not at 408 nm.
P 61: Stokes shifts must be presented in absolute energy units, such as eV and cm^{-1} but not in nm. This is not clear and cannot be compared.
P 61: Molar extinction coefficients should not be given with so many valid digits, and the precision of their determination is much lower.
P 62: PAGE results should have consistent descriptions across the chapters (either +/-/* or numbers)
P 63: Page instead of PAGE
P 66: wrong description of Fig 25: lane 6 and lane 7 should both refer to Fig 24A
P 67: Absorption spectra in Figure 26A have a line break at ~350 nm
P 76: Figure 36 should also contain the signal of dsDNA before the addition of histone (from Figure 35A)
P 77: Figure 37: it would be nice to show the real data of the state before the addition of proteamine, fully titrated and after 0.7 eq of heparin to be sure the fluorescence went to its initial value.
P 82: produt instead of product
P 82: Melting curve analysis is shown in Figure 42 D,C instead of Figure 42 D,E.
P 84: full stop is missing at the end of Chapter 3.3.1.
P 88: does λ_{abs} refer to the wavelength of absorption maximum? It is not specified.
P 93: the text says that $\mathbf{dC^{TO}}$ is non-fluorescent, but p 99 shows its fluorescence lifetime
P 94: Figure 50: H2O instead of H_2O
P 102 "thus emits fluorescent signal" instead of "thus emit fluorescent signal"
P 105: replication[178]. instead of replication.[178]
P 113: Figure 65: blue and red columns are not assigned in the description.
P 112: cps instead of cP; cps usually states "counts per second"
P 112: "Rotation between phenyl ring and BODIPY" instead of "rotation of the phenyl ring attached to BODIPY".
P 112: BODIPY vs bodipy
P 115: "show that": line break is placed incorrectly.
P 118: The text states that the absorption maximum was slightly higher. It refers to its wavelength and not the intensity.
P 123: title: phyotophysical instead of photophysical

Besides this, I attach a list of my comments and remarks that should be discussed at the disputation:

P 39: Please, explain the sensing of G-quadruplex formation shown in Figure 9C. The text does not explain this, and the emission intensities are confusing.
P 49: How does QY_{fl} of $\mathbf{dC^{Trp}}$ compare to cyan fluorescent protein?
P 95: Explain the viscosity units cP. Should the fluorescence quantum yield linearly depend on the cP scale?
P 98: Explain the behaviour of rtPCR in Figure 54.
P 100: the results in Figure 56 are very confusing. Please, explain the trends.
P 105: Why do dead cells have a longer lifetime in all compartments? How were the cells killed? Could this be caused by some interaction with the reagent? Have you performed any control experiments to find out what is happening there?

Instead of dividing into chapters by the respective fluorophore, the text would benefit from a separate discussion of spectroscopic, biochemical and application properties of fluorescently labelled ONs across the broad spectrum of prepared fluorescent labels. I like the description of the results and the amount of synthetic, spectroscopic, and biochemical work described in this thesis. While the research efforts are beyond state of the art and exciting results have been obtained, the lack of discussion that sometimes only touches the subject without any rationalisation based on the available literature hurts the otherwise high quality of the text.

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Besides the listed errors and the partial lack of discussion of some experimental findings, I find this thesis to be highly scientifically relevant, and I declare that it demonstrates the candidate's potential to perform high-quality scientific work across various scientific fields. Therefore, **I recommend the thesis to be accepted** by the Faculty of Science of Charles University committee.

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