

Referee Report for the Ph.D. Thesis of Mr. Pedro Güixens Gallardo entitled**Synthesis and delivery of novel fluorescently-labelled nucleotides and their nucleic acids for bio-analytical applications**

In his dissertation work, Mr. Gallardo presents his research aimed to the design, synthesis and study of modified nucleotides labeled with fluorophores and related nucleic acids for bio-analytical applications and their delivery into cells.

Several projects are described in the thesis. Oligonucleotides with *ortho* twisted intercalating nucleic acid have been developed and used as templates in the enzymatic primer extension. Two types of fluorescent nucleoside have been designed: a sterically hindered BODIPY-containing nucleotide served as a viscosity-dependent fluorescence probe, and thiophene-linked tetramethylBODIPY-labeled nucleotide as a viscosity-sensitive oligonucleotide probe of hybridization and protein–DNA interactions. In addition, a fluorescent nucleotide with solvatochromic properties was designed to monitor DNA-binding proteins interactions, and finally, transport of fluorescent nucleotide and oligonucleotide analogs was investigated.

The text is divided into three major chapters. The Introduction acquaints the reader with the necessary biological and photophysical terms and the methods and strategies that were used in the study, such as design of fluorescent nucleosides, time-correlated single-photon counting, or the incorporation of nucleosides into cells. The chapter on Results and Discussion provides the reader a comprehensive information about all projects studied under the tutelage of Professor Michal Hocek. The Experimental Part is an extensive and detailed information which testifies to the depth and diversity of the research performed by Mr. Gallardo.

A large amount of original and state-of-the-art results and discussion demonstrates erudite, professional and sophisticated combination of vision and experiment. The thesis shows a noteworthy depth of understanding and detail. In general, the text is formally well organized and written in an eloquent and logical style.

The English is idiomatic and clear, although some marginal and formal errors and inaccurate statements (terms) can be found throughout the text. It is also noted that the results described in this thesis have already been published in three highly-impacted journals.

Here, the following comments, questions, and truly minor issues may deserve the attention during the defense.

Questions

- Page 19, 2nd paragraph: Why is a chromophore defined by its absorption above 200 nm?
- Figure 16, right: What are the conditions and limitations of this quenching?
- Page 58: Is there any evidence for dye aggregation? Please explain in detail the variations of the fluorescent quantum yields shown in Table 2. The spectra of dA^{mBdp} and dU^{mBdp} seem to be very similar (page 221); but what about the TP derivatives in buffer?
- Page 67: Please explain the large Stokes shifts observed.
- Figure 53: How were the spectra normalized (see the spectrum in ACN)?
- Page 70: Please explain the term “supramolecular process” mentioned there.
- Table 6: Was the tendency to aggregate of these compounds tested under all relevant conditions?
- Page 71: Is this hypothesized interaction really of a π - π character? Is there any other alternative? In addition, aggregation-induced emission can be tested.
- Why are the lifetime symbols shown as $\langle \tau \rangle$?
- Figure 77: Was the fluorescence quantum yield measured?
- Page 104: What is the evidence of the keto/enol tautomeric equilibrium for **20**?

- Page 110: Please explain how “the energy of activation required for the isomerisation” is related to the photon energy (it may not be as trivial as it sounds).
- Figure 103: Some of the spectra may be composed of two bands. Is there any explanation for that?

Examples of *minor inaccuracies and unusual terms*: “half lifetime time”, “phenolic alcohol”, “*para*-oriented product”, “Stoke shift”, “molar extinction coefficient” (an obsolete term). “Polarity can be described as a sum of intermolecular forces as dipole-dipole but...”, “the efficiency of emitting photons ...”, “The fluorescence lifetime values of the fluorophores are not comparable since molecules that have been longer in the excited state (S1) are prone to quenching processes.”. “If the methylation is present in the *ortho* position ...”, “The electrophilic nitrogen attacked to the highly electrophilic trifluoroborate etherate.”, “UV light did not affect the NMR of ...”,

In conclusion, I state that this excellent dissertation work clearly merits our acknowledgment. I am pleased to give a full support to the thesis application of Mr. **Pedro Güixens Gallardo**, and I propose that the thesis **is accepted without reserve**.



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