Interaction of ROX and ssGreen Fluorophores Affects Visualization of Gel Electrophoresis Results

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Abstract. Fluorescent molecules have found wide application in biological and medical research: from flow cytometry and confocal microscopy to ELISA and PCR methods. Understanding the properties of fluorophores and their interactions is an integral part of developing new research methods in a wide range of scientific fields. In this paper, we present the results of the analysis of the interaction of carboxy-X-rhodamine (ROX) and the intercalating dye of the green spectrum for single-stranded DNA and RNA molecules – ssGreen. It was found that the presence of ssGreen in the agarose gel visually gives a faint enhance to the rhodamine signal compared to the gel without ssGreen. Moreover, the absence of green spectrum luminescence (quenching) was observed in the oligonucleotide with rhodamine in its composition, in contrast to the PCR primer without fluorophores in its composition. The proposed FRET phenomenon is not supported by the results of quantum mechanism calculations, which requires further study.

1 Introduction

Fluorescent molecules have found wide application in biological and medical research: from flow cytometry and confocal microscopy to ELISA and PCR methods. Studying the properties of fluorophores and their interactions allows their combinations to be used to develop new methods, including PCR using FRET probes for genotyping [1], detection of microbial pathogens [2-4] and a wide variety of analytes [5-7], fluorescence *in situ* hybridization [8], flow cytometry based on FRET (Förster energy transfer) [9] and confocal microscopy of DNA conformation [10].

Fluorophores have a wide variety of excitation and emission spectra and have different chemical classifications (cyanines, fluoresceins, etc.). The fluorophores FAM (fluorescein), HEX (hexachlorofluorescein), ROX (carboxy-X-rhodamine), Cy5 (cyanine5), and Cy5.5 (cyanine5.5) are among the most popular reporter molecules for fluorescent detection of amplification products for real-time PCR [11]. These molecules, along with their spectral analogs, are used, for example, in TaqMan probes paired with fluorescence quenchers.

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However, there are combinations such as TAMRA and FAM, where the TAMRA fluorophore is a FAM quencher [12]. Such FRET interactions of fluorophores are particularly interesting both from the point of view of fundamental chemistry and from the point of view of developing new methods for molecular diagnostics [13].

Studies on the Förster energy transfer of the ROX fluorophore have shown that it is often used as a FAM quencher. Thus, in DNA sequencing, FRET primers with FAM as a donor and ROX as an acceptor demonstrated a better analysis result compared to primers with single fluorophores [14, 15]. It is worth noting that the peak of fluorescein emission coincides with the maximum absorption of rhodamine. It has been shown that as ROX being placed away from FAM during oligonucleotide hybridization with excitation at 490 nm, fluorescence with a peak at ~590 nm becomes less intense [16], i.e. energy transfer occurs for these two molecules: at 490 nm, FAM is excited, its emission leads to ROX excitation, and, ultimately, ROX emission is observed in the peak of the spectrum corresponding to it.

In this paper, we present the results of the analysis of the interaction of carboxy-X-rhodamine (ROX) and the intercalating green dye for single-stranded DNA and RNA molecules – ssGreen.

2 Materials and Methods

To visualize the interaction of ROX and ssGreen fluorophores, we conducted experiments using agarose gel electrophoresis with and without an intercalating dye.

The oligonucleotide containing ROX was used to study the FRET phenomenon with ssGreen. It had the following structure: [Azide-NHS-AminoC6]tt[ROX5-dT-C]ttt[ROX5-Pro-C]. 4% Agarose gel was prepared using agarose LE 2 (Lonza Rockland, Inc.) and TAE buffer (Eurogen, CJSC). 5 µl of ssGreen 10000x (Lumiprobe, LTD) was added only after gel boiling. In its chemical structure, this dye is very close to SYBR Green II (Fig. 1). Electrophoresis of the DNA ladder and the oligonucleotide containing ROX was carried out for 45 minutes at 75 mA and 110 V. The electrophoresis conditions were the same for both the gel with and without ssGreen. The DNA length marker was visualized by adding Eva488 20x (Lumiprobe, LTD) before loading into the gel. The gel was visualized using a UV transilluminator ChemiDoc XRS+ System (Bio-Rad Laboratories, LLC) with a peak at 302 nm.

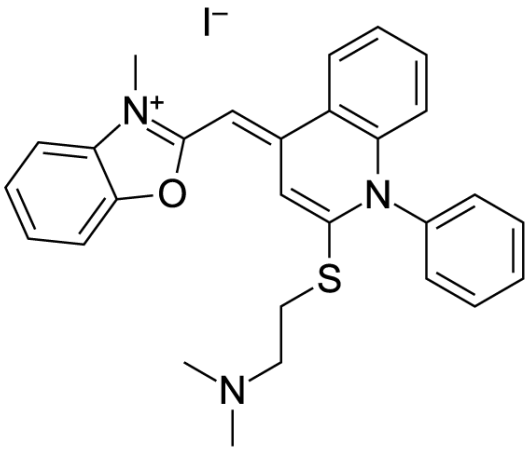

Fig. 1. Chemical structure of the fluorescent intercalating dye of single-chain molecules ssGreen.

After observing interaction of fluorophores in agarose gel, we calculated the quantum interactions of these molecules. Quantum-chemical calculations were performed using the ORCA version 5.0.4 program [17]. The energy of the frontier molecular orbitals was calculated using the DFT method. The hybrid functional B3LYP and the basis set def2-TZVP [18] were used to calculate the energy of the molecular orbitals.

3 Results

As a result of gel electrophoresis of the oligonucleotide containing ROX to study interactions with the fluorophore ssGreen, it was revealed that the presence of ssGreen in the agarose gel visually somewhat enhances the rhodamine signal (Fig. 2, right) compared to the gel without ssGreen (Fig. 2, left).

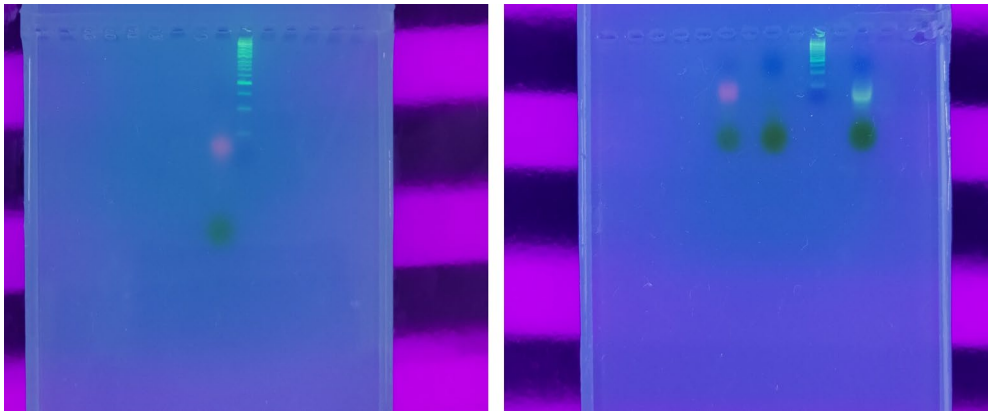

Fig. 2. Rhodamine luminescence intensity in the presence (right) and without ssGreen (left) in an agarose gel. The figure on the right shows, in order: oligonucleotide with ROX in its composition, buffer for application to the gel (contamination check), DNA ladder, random primer for PCR without fluorophores in its composition (ssGreen quality check).

Moreover, the absence of green spectrum luminescence was noted in the oligonucleotide with rhodamine in its composition, in contrast to the PCR primer without fluorophores in it (Fig. 2, right, last track). The presumably observed quenching phenomena sparked our interest, and then we carried out quantum calculations in the system of these two molecules.

Quenching of fluorescence of the ssGreen dye in the presence of a ROX-modified oligonucleotide was recorded visually. This phenomenon can be explained with the FRET effect, Dexter electron transfer, or with the pi-stacking of dye molecules. For the FRET effect or Dexter electron transfer to occur, the highest occupied molecular orbital (HOMO) of the donor molecule must be located higher on the energy diagram relative to the lowest unoccupied molecular orbital (LUMO) of the acceptor molecule. Based on the quantum mechanical calculations performed, an energy diagram of the boundary molecular orbitals of the dye molecules was constructed (Fig. 3).

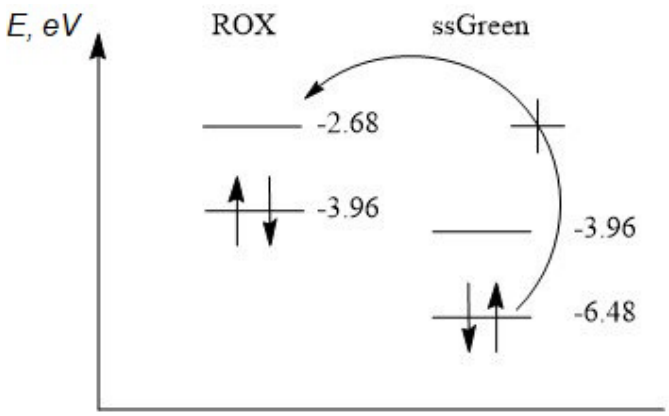

Fig. 3. Energy diagram of frontier molecular orbitals of ROX and ssGreen dyes.

The donor is a dye molecule whose fluorescence is suppressed, in this case it is ssGreen. As can be seen from the constructed diagram, the HOMO orbital of the donor molecule is located significantly below the LUMO of the acceptor molecule. The implementation of FRET or Dexter electron transfer mechanisms seems no to be the case for this particular system of fluorophores. The observed phenomena can be explained by pi-stacking of the dye molecules.

4 Conclusion

Understanding the properties of fluorophores is an integral part of developing new research methods using them in a variety of scientific fields. Green and orange fluorophores are a popular pair in a variety of detection systems: from PCR to flow cytometry. We have found a putative quenching interaction of fluorophores, which should be taken into account in the future when used, for example, in Sanger sequencing or fragment analysis. It should be noted that ssGreen, as a fluorophore with an emission peak in the absorption peak of carboxy-X-rhodamine, is a donor for it in the system of these two molecules. Proposed FRET interactions coincide with previously described data in the literature, but the results of calculations of quantum mechanisms contradict the results of experimental studies, which requires further study.

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