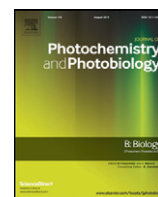




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Sequence specific recognition of ssDNA by fluorophore 3-hydroxyflavone

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ABSTRACT

A fully water soluble 3-hydroxyflavone (3HF) derivative, N-(3-hydroxy-4'-flavonyl)-N,N,N-trimethylammonium sulfate (3HFNMe3) was synthesized. Investigation of its emissions at varying wavelengths revealed that it had three emission bands of normal (N^*), anionic (A^*) and tautomeric (T^*), in ultrapure water. Recognition of single-stranded ten ssDNA chains, having different nucleotide sequences was studied, using the ratiometric change of the intensities of the two bands (A^*/T^*), depending upon the varying environment of the 3HFNMe3 with different ssDNA chains. Addition of the ssDNA chains to the 3HFNMe3 solution caused gradual quenching of the A^* band and had almost no effect on the T^* band. As the ratios of the two bands (A^*/T^*) vs increasing amount of the ssDNAs generated characteristic curves for each ssDNA chain, it became possible to identify the chains with their characteristic curves.

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1. Introduction

Designing DNA probes for detecting DNA chains, having varying sequences is a great challenge [1]. The motivation for DNA detection is due to its widespread applications in various fields such as DNA diagnostics, gene analysis and detection of genetic mutations, which may lead to the gene analysis before symptom(s) of a disease appears.

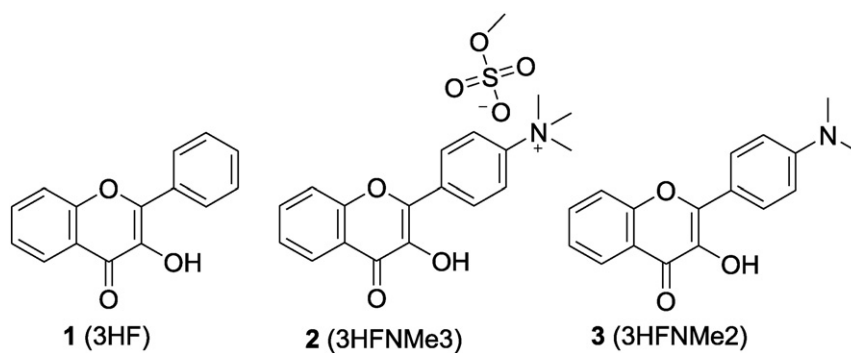
Majority of the probes synthesized so far depends on designing complementary probes for targeted DNA sequences to allow them to hybridize with the probe. Such a process could be performed in solution or probe could be presented on a solid support. Their detection could be electrochemical [2,3] or optical [4–8]. However, in majority of the cases, nucleic acid is covalently labeled by a fluorescent probe and the detection is studied by fluorescence resonance energy transfer (FRET) or fluorescence quenching. On the other hand, detection of DNA through the interactions of positively charged fluorophore with negative charges of DNA is rare [9].

3-Hydroxyflavones (3HF) **1** (Scheme 1) have unique photophysical properties, which exhibit well-resolved and intense two fluorescence bands in the visible region, resulting from excited-state intramolecular proton transfer (ESIPT) (Scheme 2), i.e. the initially excited normal form (N^*) and the tautomeric form (T^*) [10–13]. Their ratios are highly sensitive to the environment such as polarity and hydrogen bonding

perturbations of the solvents. As the ratio between two bands depends on the microenvironment of 3HF, not on its concentration or the instrument settings, this property provides 3HF with an important advantage over conventional single-band dyes [14–16]. Information could be obtained about the microenvironment of 3HF with the change of the ratios of its two emission bands (I_{N^*}/I_{T^*}) and this ratio could be specific for each environment. Examples have already been reported for sensing lipid bilayers, cell membranes, proteins and peptides using 3HF as a sensor [17–19]. Concerning the use of 3HF as a two-band switchable fluorescence sensor for the interactions of DNA, 3HF as a nucleobase mimic has been demonstrated [4]. Double- and single-stranded DNAs were sensed through their characteristic effects on binding to 3HF, possessing polycationic spermine [20]. It was disclosed that while on binding to a double-stranded DNA, emission bands of the 3HF changed up to 16-fold, only moderate changes in the dual emission on binding to a single-stranded DNA was observed. In a separate study, ratiometric change in two emission bands of 3HF was used as a tool to distinguish different peptide-ODN complexes, [21] and using the similar technology, a series of 3HFs were synthesized and successfully applied to identify various peptide-ODN complexes [22]. Moreover, fisetin, which is a 3HF derivative, was employed for recognition of DNA nucleotide based on selective abasic site binding [23].

In this study, a novel fully water soluble 3-hydroxyflavone (3HF) derivative, N-(3-hydroxy-4'-flavonyl)-N,N,N-trimethylammonium sulfate (3HFNMe3) **2** (Scheme 1), which had emission bands of normal (N^*), anionic (A^*) and tautomeric (T^*), in ultrapure water, was employed as

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Scheme 1. Structures of 3-hydroxyflavone and its derivatives 3HFNMe3 **2** and 3HFNMe2 **3**.

a fluorophore for recognition of single-stranded DNAs (ssDNA), having varying nucleotide chains. The study discloses that gradual addition of ssDNA to the solution of the fluorophore, 3HFNMe3, resulted in obtaining characteristic I_{A^-}/I_{T^+} curve, rather than conventional I_{N^+}/I_{T^+} ratio, for each single-stranded DNA chain.

2. Experimental

All reagents were purchased from Aldrich, Acros and Merck, and were used without further purification. All the solvents used in the syntheses were technical grade and freshly distilled prior to use. The water used for the measurements had an ultrapure grade (distilled from Millipore Milli-Q Academic). The single-stranded DNA chains were obtained from Metabion International AG, Germany. UV–Vis and fluorescence measurements were studied on HITACHI U-0080D and HITACHI F-4500, respectively. ^1H and ^{13}C NMR spectra were recorded on a Varian 600 spectrometer. Proton and carbon chemical shifts were reported in parts per million downfield from tetramethylsilane, TMS. Mass spectra were recorded on ThermoLCQ-Deca ion trap mass instruments.

2.1. Synthesis of *N*-(3-hydroxy-4'-flavonyl)-*N,N,N*-trimethylammonium sulfate **2** (3HFNMe3)

Dimethylamine 3-hydroxyflavone (3HFNMe2) **3** was synthesized following the literature procedure [24]. A solution of **3** (0.37 g, 1.3 mmol) and dimethylsulfate (6.55 mmol, 0.62 ml) in THF was refluxed for 24 h. The precipitate was filtered after the mixture was

reached room temperature. The crude solid was purified by Soxhlet extraction, using dichloromethane as solvent, which yielded *N*-(3-hydroxy-4'-flavonyl)-*N,N,N*-trimethylammonium sulfate (3HFNMe3) **2** as a white solid (0.122 g, 23%). ^1H NMR (600 MHz, D_2O) δ (ppm) 8.11 (d, 2 H, $J = 8.4$ Hz), 7.82 (d, 2 H, $J = 7.6$ Hz), 7.71 (d, 2 H, $J = 8.4$ Hz), 7.61 (t, 1 H, $J = 7.6$ Hz, $J = 7.5$ Hz), 7.44 (d, 1 H, $J = 7.6$ Hz), 7.25 (t, 1 H, $J = 7.6$ Hz, $J = 7.5$ Hz), 5.32 (s, 9 H), 3.61 (s, 1 H), 3.55 (s, 3 H); ^{13}C NMR (150 MHz, D_2O) δ (ppm) 157.2, 134.5, 132.1, 129.5, 129.4, 129.3, 125.1, 124.9, 124.4, 124.3, 119.8, 119.7, 118.3, 56.8, 53.8; Found C, 55.78, H, 5.35, N, 3.60, Calculated C, 56.03, H, 5.22, N, 3.44; m/z 296 M^+ .

2.2. Fluorescence Measurements

The single-stranded DNA chains comprising 25 Cytosines (d(C)25), 25 Guanines (d(G)25), 25 Adenines (d(A)25), 25 Thymines (d(T)25), 15 Cytosines and 5 Adenines (d(C)15-d(A)5), 10 Cytosines and 10 Adenines (d(C)10-d(A)10), 5 Cytosines and 15 Adenines (d(C)5-d(A)15), 15 Thymines and 5 Guanines (d(T)15-d(G)5), 10 Thymines and 10 Guanines (d(T)10-d(G)10) and 5 Thymines and 15 Guanines (d(T)5-d(G)15) were applied for the measurements.

From a stock solution of **2** (3HFNMe3), dissolved in ultrapure water (2.1 mg, 2 ml, 2.5×10^{-3} M), was transferred 200 μl into a quartz cell ($1 \times 1 \times 3$ cm) and diluted to 2 ml with ultrapure water. Fluorescence emission was then recorded after each addition the ssDNA solution (2 μl , 20 nmol/ml). The ratio of A^* and T^* bands was calculated and a graph, having A^*/T^* ratio vs DNA concentration was plotted. Each experiment was repeated five times to understand the repeatability of the results, which gave the same results.

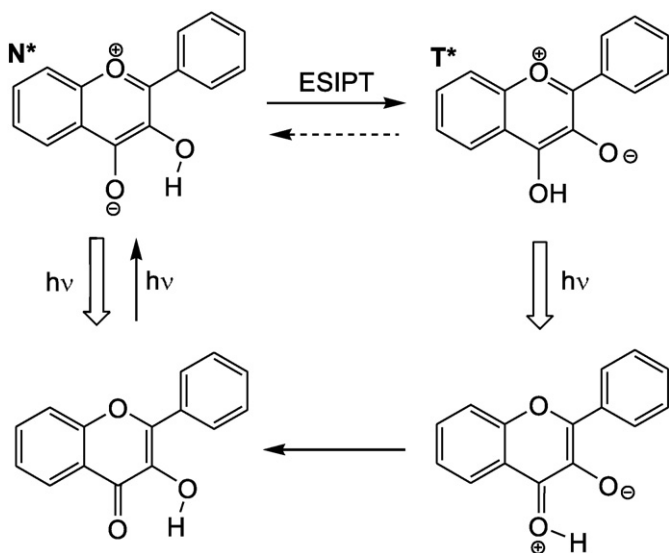
3. Results and Discussion

3.1. Synthesis

The precursor, dimethylamino-3-hydroxyflavone (HFNMe2) **3**, to obtain its salt form *N*-(3-hydroxy-4'-flavonyl)-*N,N,N*-trimethylammonium sulfate (3HFNMe3) **2**, was synthesized applying the literature procedure (Scheme 3) [24]. Its methylation with dimethylsulfate and then purification of the product with Soxhlet extraction successfully gave the product **2** in 23% yield.

3.2. Spectroscopic Measurements

UV measurement of 3HFNMe3 **2**, methyl salt of 3HFNMe2 **3**, having maximum at 400 nm in dichloromethane, [24] displayed two maxima at 305 and 345 nm in ultra-pure water (Fig. 1). While 3HFNMe2 had a broad peak between 330 and 450 nm, its methyl salt 3HFNMe3 had two maxima covering a shorter area of 280–390 nm. Excitation of 3HFNMe3 at maximum 345 nm produced two emissions at 405 (weak) and 520 nm (strong) as N^* and T^* bands (Fig. 1, A). As for ratiometric study, such an emission was not suitable since the intensity



Scheme 2. ESIPIT reactions of 3HF.

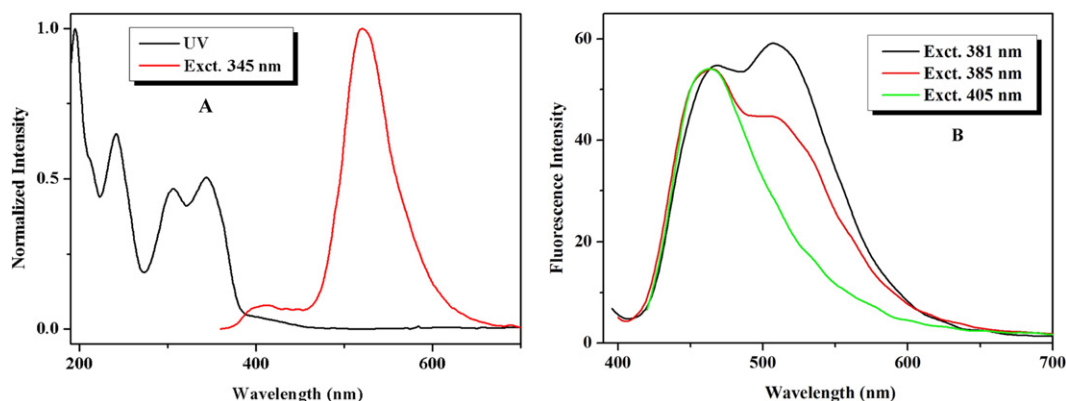
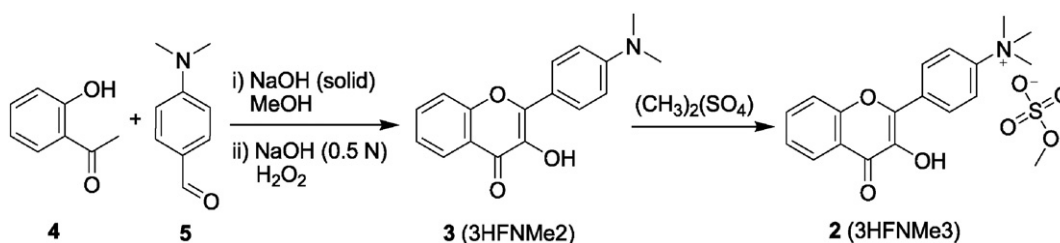


Fig. 1. Absorption and fluorescence spectra of 3HFNMe3 2 in ultra-pure water.

of the N^* band was too weak. Thus, an excitation screening was conducted, which displayed that the two best bands for a ratiometric study were available with the excitation at 381 nm, as the excitations at various wavelengths such as 385 and 405 nm caused the T^* band

became weaker and then disappear (Fig. 1, B). Although the intensities of the both emissions (ex. 381 nm) were not too strong, they were found to be enough for a ratiometric study.

A close investigation of the emission bands of the excitations at 345 and 381 revealed that their first emission bands appeared at different wavelengths of 405 and 460 nm, respectively, as their second emissions were the same, 520 nm, which indicated that a different ESIPT mechanism for 3HFNMe3 was taking place. As 3HFNMe3 had a positive charge on nitrogen attached to the para of the phenyl group, proton of the hydroxyl group could be acidic enough to be deprotonated to generate an anion. Indeed, pH measurement and computational study indicated that 3HFNMe3 had pH of 5 and 5.77, respectively. Regarding the location of the T^* band of 3HFNMe3, compare with the T^* band of 2-(4-(dialkylamino)phenyl)-3-hydroxy-4H-chromen-4-one **6** (Scheme 4), emissions of which studied in depth [18], while 3HFNMe3 had T^* emission at shorter wavelength of 520 nm, dialkyl-3HF had T^* emission at longer wavelength of around 580 nm. Such a difference arises from the participation of free electrons of amino group into conjugation, forming an $H-N^*$ emission at shorter wavelength corresponding the T^* emission of our quaternized 3HFNMe3. On the other hand, as the amino group of 3HFNMe3 did not have any free

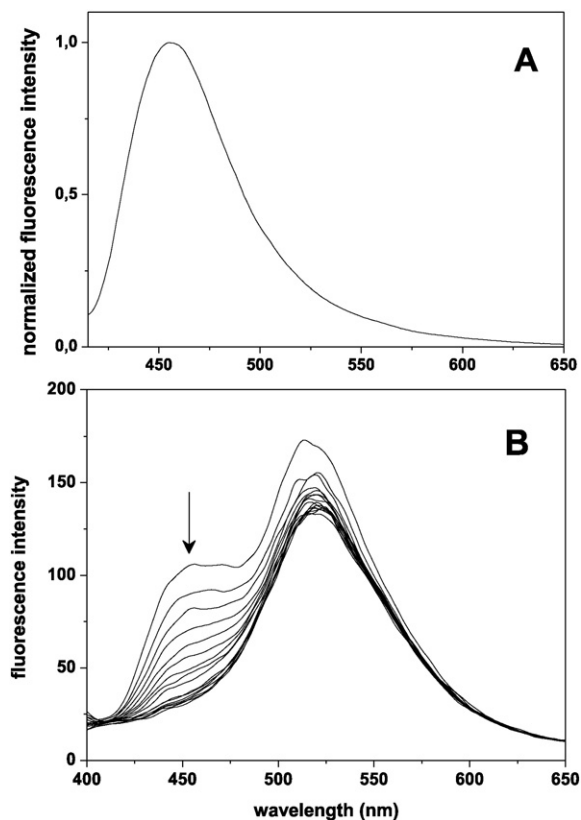
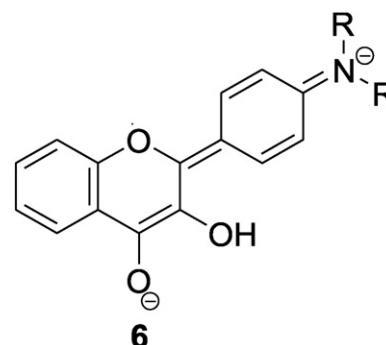


Fig. 2. Emissions of 3HFNMe3 2 A) in water; B) in ultra pure water (quenching of A^* band with the addition of ssDNA, Exct. 381 nm, pH = 5).



Scheme 4. Resonance of N,N-dialkylamino-3HF.

electrons, the group is quaternized with methylation, such an emission ($H-N^*$) could not be expected. Thus, it was decided that while the N^* (405 nm) and T^* (520 nm) emission bands of 3HFNMe3 are in good agreement with the emissions indicated by Kasha in his extensive study of hydrated 3HF forms, [25] a new emission at 460 nm could belong to anionic form **11** (A^*) of 3HFNMe3. While the conventional ESIPT reaction of 3HFNMe3 **2** was observed with N^* **7** and T^* **8**, excited forms of **N2** and **T9** ground states, respectively, a proton abstraction from 3HFNMe3 **2** generated the anionic form **A** **10** and its excitation at 381 nm produced the excited anion A^* **11**, emission of which gave the anionic emission peak at 460 nm (Scheme 5). As the ratiometric study was performed with excitation at 381, the concerned emissions are A^* and T^* , which means that the ratiometric measurement in this study belongs to A^*/T^* ratio.

Initially, phosphate buffer was planned to use in all measurements. Unfortunately, addition of small amount of buffer to the solution resulted in quenching the A^* band of the sensor (See the Supporting Information). Then, as the fluorophore was found to be so sensitive to the buffer, all the measurements were decided to be conducted in solution without buffer. Indeed, it was noted that pH of the solutions in all the measurements did not change. pH of the pure water was measured to be 7.5, which became 5 with the addition of the probe 3HFNMe3 **2** and remained at 5 in all measurements. The change of pH from 7.5 to 5 could be due to the presence of positive charge on the nitrogen atom, which increases its electronegativity, causing the hydroxyl proton to be more acidic. In order to clarify this, a computational study was decided to be conducted.

3.3. Computational Study

To shed more light on the relative acidity of dimethylamine, 3HFNMe2, **3** and its quaternized, tetramethylamine 3HFNMe3, **2** forms, their pK_a values were predicted with the isodesmic reaction, applying methodology reported in the literature [26]. The experimental pK_a value of phenol (9.98) was taken as a reference. DFT calculations were carried out using the Gaussian 09 program [27] Becke's three-parameter exchange functional (B3) [28,29] was employed with the Lee–Yang–Parr correlation functional (LYP) [30] as implemented in Gaussian 09 together with People's split-valence 6-311++G(d,p) basis

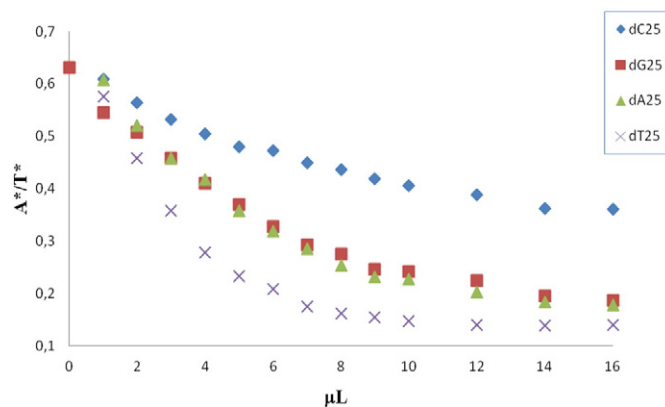
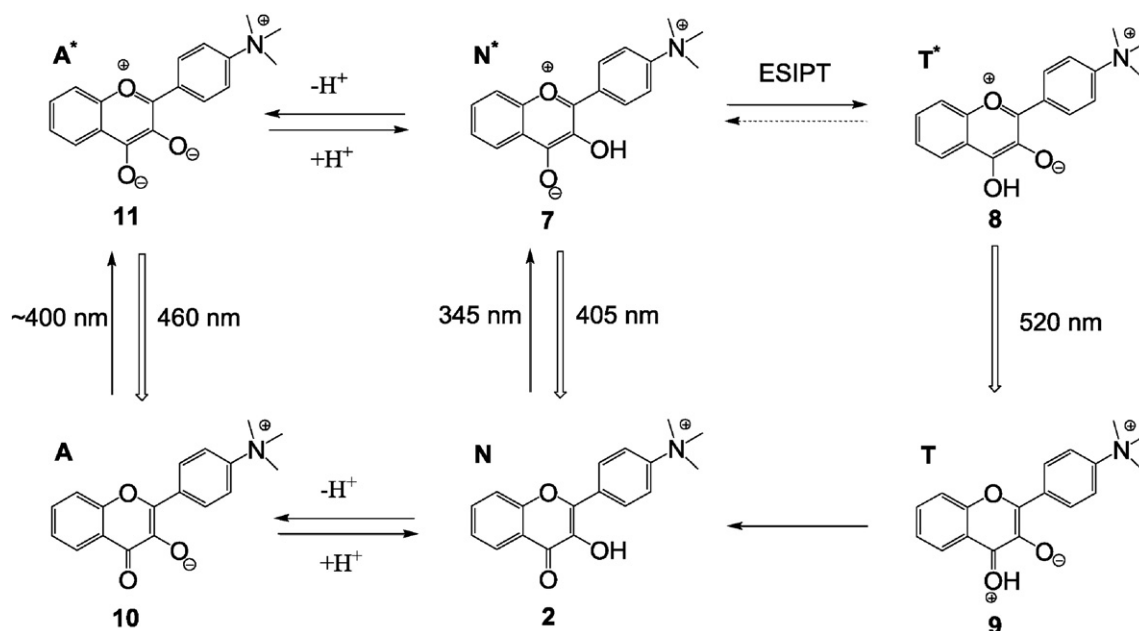


Fig. 3. Emissions of **2** with the addition of d(C)25, d(G)25, d(A)25 and d(T)25 (Exct. 381 nm, pH = 5).

set. The minima of the calculated structures were verified by analyzing the harmonic vibrational frequencies, using analytical second derivatives, which have $\text{nimag} = 0$. Solvent effect was included using water in the SMD solvation model [31]. Computational studies estimated the pK_a values of 3HFNMe2 and 3HFNMe3 as 8.94 and 5.77, respectively, indicating the fact that aqueous 3HFNMe3 is substantially more acidic than 3HFNMe2. Thus, the experimental pK_a of 3HFNMe3 is in good agreement with its calculated value.

3.4. DNA Studies

Although some of the water soluble 3HF derivatives consisted of quaternized amine groups [32], they were not informative enough as the fluorophore had only one emission band (N^*), and could not be employed as sensor for ratiometric measurements of the band intensities (I_{N^*}/I_{T^*}). When we performed fluorescence measurements with the fluorophore **2** (3HFNMe3), having quaternized amine group, in tap water and distilled water, same results, *i.e.* single emission band, corresponding to anionic emission, A^* , was obtained (Fig. 2A). Surprisingly, when the experiment was repeated in ultrapure water, two



Scheme 5. Normal (N^*), anionic (A^*) and tautomeric (T^*) emission mechanisms of 3HFNMe3.

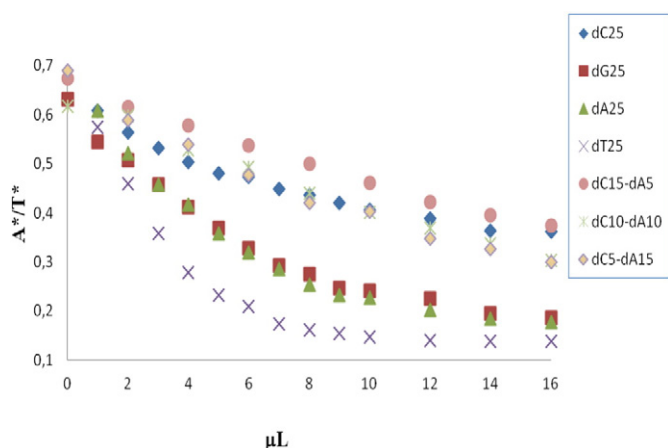


Fig. 4. Emissions of **2** after addition of d(C)25, d(G)25, d(A)25, d(T)25, d(C)15-d(A)5, d(C)10-d(A)10 and d(C)5-d(A)15 (Exct. 381 nm, pH = 5).

emission bands of A^* and T^* appeared (Fig. 2B), and gradual addition of ssDNA to the fluorophore solution resulted in the quenching of the A^* band.

Considering that the A^*/T^* ratio could change depending upon the nucleotide sequence of the ssDNA, various ssDNA chains were employed to obtain characteristic A^*/T^* ratio for each sequence. Then, gradual addition of ssDNA solutions into the 3HFNMe3 **2** solution, both in ultra pure water, resulted in varying A^*/T^* ratios with the nature of ssDNA chain (Fig. 3). While A^*/T^* curve of d(C)25 was calculated to be the highest, d(T)25 had the lowest curve. The curves of A^*/T^* ratios of d(A)25 and d(G)25, which took place between the highest and the lowest curves of d(C)25 and d(T)25, respectively, followed almost the same line. As it is known that 3HFs are very sensitive to their environment, possible explanation could be that the ssDNA chains had varying interactions with 3HFNMe3, due to their different chemical structures and conformations, which affected the fluorescence intensities. Then, the ssDNA chains, possessing two different nucleotides in different ratios were subjected to the recognition by 3HFNMe3. The curves of d(C)15-d(A)5 (Fig. 4), d(C)10-d(A)10, d(C)5-d(A)15 (Figs. 4 and 5), d(T)15-d(G)5, d(T)10-d(G)10 and d(T)5-d(G)15 (Fig. 5) took part around the curve obtained from the ssDNA chain d(C)25 (Fig. 6), which may imply that the mixture of single-stranded DNA chains have closer

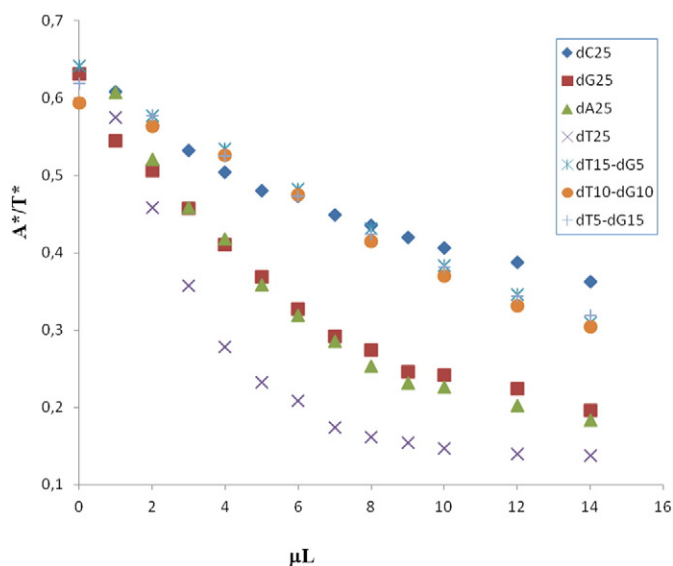


Fig. 5. Emissions of **2** after addition of d(C)25, d(G)25, d(A)25, d(T)25, d(T)15-d(G)5, d(T)10-d(G)10 and d(T)5-d(G)15 (Exct. 381 nm, pH = 5).

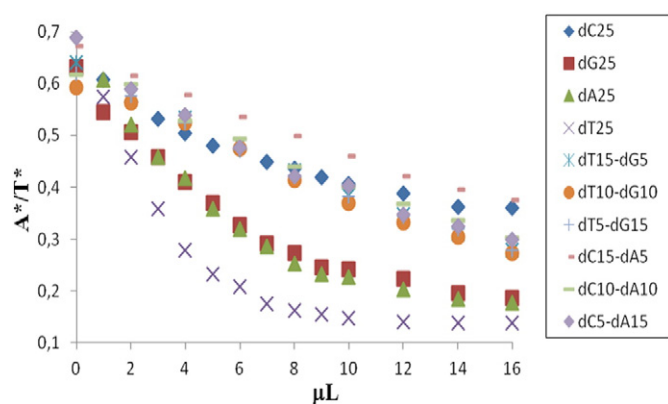


Fig. 6. Emission of **2** with all the DNA chains (Exct. 381 nm, pH = 5).

conformation with d(C)25. Considering that the ssDNA chains have small structural differences, such recognition is an important result.

4. Conclusion

A water soluble fluorescent sensor has been developed to identify single-stranded DNAs having different nucleotide sequences. This fluorophore had three emission bands of normal (N^*), anionic (A^*) and tautomer (T^*) at 405, 460 and 520 nm, respectively. The anionic emission arose from the anionic form of 3HFNMe3, which had a slightly acidic hydroxyl proton having pH of 5. The ratio of A^*/T^* was found to be suitable for a ratiometric study and the results presented herein indicated that the flavone 3HFNMe3 is a potentially important sensor for identification of various chemical conformations such as single-stranded DNAs. Although the results of this study are moderate, they indicate that ratiometric technique of 3HF is an important tool to identify various ssDNA chains. In this study, only single-stranded DNA chains were attempted to identify, and the results have been reported. As a continuation of this study, identification of various biological molecules, particularly water soluble ounces, such as double-stranded DNA chains will be conducted.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jphotobiol.2015.10.019>.

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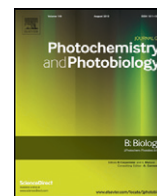
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Corrigendum

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The authors regret that there was an error in the last name of one of the authors. Please consider the corrected name as “Muslum Akgoz”.



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