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ORIGINAL PAPER

The Fluorescence Properties and Lifetime Study of G-quadruplexes Single- and Double-labeled with Pyrene

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Abstract We report steady state fluorescence and lifetime emission studies of d(GGTTGGTGTGGTTGG) (TBA) and d (GGGTTAGGGTTAGGGTTAGGG) (Htelom) oligonucleotides labeled with pyrene through a 3-aminopropyl linker. Such G-rich sequences are able to self-assemble into G-quadruplexes, especially in the presence of specific cations like potassium. A comparative studies with single- and double-labeled G-quadruplexes were carried out. For each probe we have measured fluorescence decays for emission wavelength of 390 and 480 nm in the varying concentration of potassium ion. We have calculated average lifetimes $<\tau>$ for every system as well as the fractional distribution α_i of emitting species.

Keywords Excimer · Fluorescence · G-quadruplex · Lifetime · Potassium probe · Pyrene

Introduction

Specific fluorescence properties of pyrene, for example ability to create fluorescent excimer, and the large number of techniques available for its detection contributed to great interest in applying pyrenyl derivatives in bioanalytical assays as well as in structural investigations of biosystems [1–9]. It is worth mentioning that by means of appearing and increasing excimer signal it was possible to monitor formation of tetramolecular G-quadruplexes [10].

Antithrombin binding aptamer sequence, d(GGTTG GTGTGGTTGG) (TBA) is one of the most often examined G-quadruplexes. It is known, that in the presence of potassium ion TBA folds into four stranded structure characterized by a chair type topology [11]. Therefore, in our current research concerning interaction between G-quadruplexes and biometals or ligands, we focused on developing oligonucleotide-based sensors, which are expected to exhibit fluorescence responses after addition of target analyte [12–17]. So far, we have proved that dual-pyrene-labeled TBA sequence is an ideal probe to monitor changes in potassium ion concentration, even in the presence of an excess of sodium [15]. Recently, we have examined the fluorescence properties of G-quadruplex probes with human telomere sequence, d(GGG $(TTAGGG)_3$) (Htelom) possessing pyrene attached to both termini [16, 17]. In this case, it appeared that diverse topologies of Htelom quadruplexes have great influence on their spectroscopic properties. The most important observation was that dual-labeled Htelom probe exhibited only negligible long-wave fluorescence and with short average lifetime in contrary to the analogous TBA-based probe.

Above mentioned results have turned our attention to studying more deeply the fluorescence features of pyrene labeled TBA and Htelom probes. For this purpose, we have attached pyrenebutanoic acid (PBA) through a 3-aminopropyl linker to 5′, 3′ or both termini of TBA and Htelom oligonucleotides (Table 1). Here we report results of the direct measurements of K^+-induced changes in their steady state fluorescence spectra as well as in decay profiles.

Experimental

Materials

Pyrene-labeled oligonucleotide probes were custom synthesized by Sigma-Genosys, Japan. A 100 μM stock solutions

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Table 1 General overview of investigated probes

Probe	Oligonucleotide	5′ label	3′ label
Py-TBA	d(GGTTGGTGTGGTTGG)	Pyrene	–
Py-TBA-Py	d(GGTTGGTGTGGTTGG)	Pyrene	Pyrene
TBA-Py	d(GGTTGGTGTGGTTGG)	–	Pyrene
Py-Htelom	d(GGGTTAGGGTTAGGG TTAGGG)	Pyrene	–
Py-Htelom-Py	d(GGGTTAGGGTTAGGG TTAGGG)	Pyrene	Pyrene
Htelom-Py	d(GGGTTAGGGTTAGGG TTAGGG)	–	Pyrene

of the probes and Tris-HCl buffer (pH 7.4) were prepared in ultrapure water (Polwater D-100UIM). The sample solution (800 μl) containing oligonucleotide probe at concentration of 1 μM in 5 mM Tris-HCl buffer (pH 7.4) and proper amount of KCl were prepared one day before spectral measurements. Each sample solutions was heated at 95 °C for 5 min. and cooled slowly to room temperature (RT) and kept in ca. 0 °C overnight.

Methods

Fluorescence emission and excitation spectra were recorded at room temperature using a JASCO spectrofluorometer. Fluorescence probes were excited at 340 nm and the emission spectra were recorded in the 380–600 nm spectral range. Fluorescence emission from the solvents was found to be insignificant. Fluorescence quantum yields were determined using pyrenebutanoic acid (PBA), 1×10^{-6} M, as a reference (relative ϕ was assumed to be 1). Fluorescence lifetimes were measured with an IBH Consultants (Glasgow, Scotland) model 5,000 fluorescence lifetime spectrometer using a laser diode as an excitation source (340± 10 nm). The fluorescence decays were collected at emission wavelength of 390 nm as well as at 480 nm. Deconvolution of the fluorescence decay curves was performed using the IBH Consultants Version 4 software. The quality of the fit

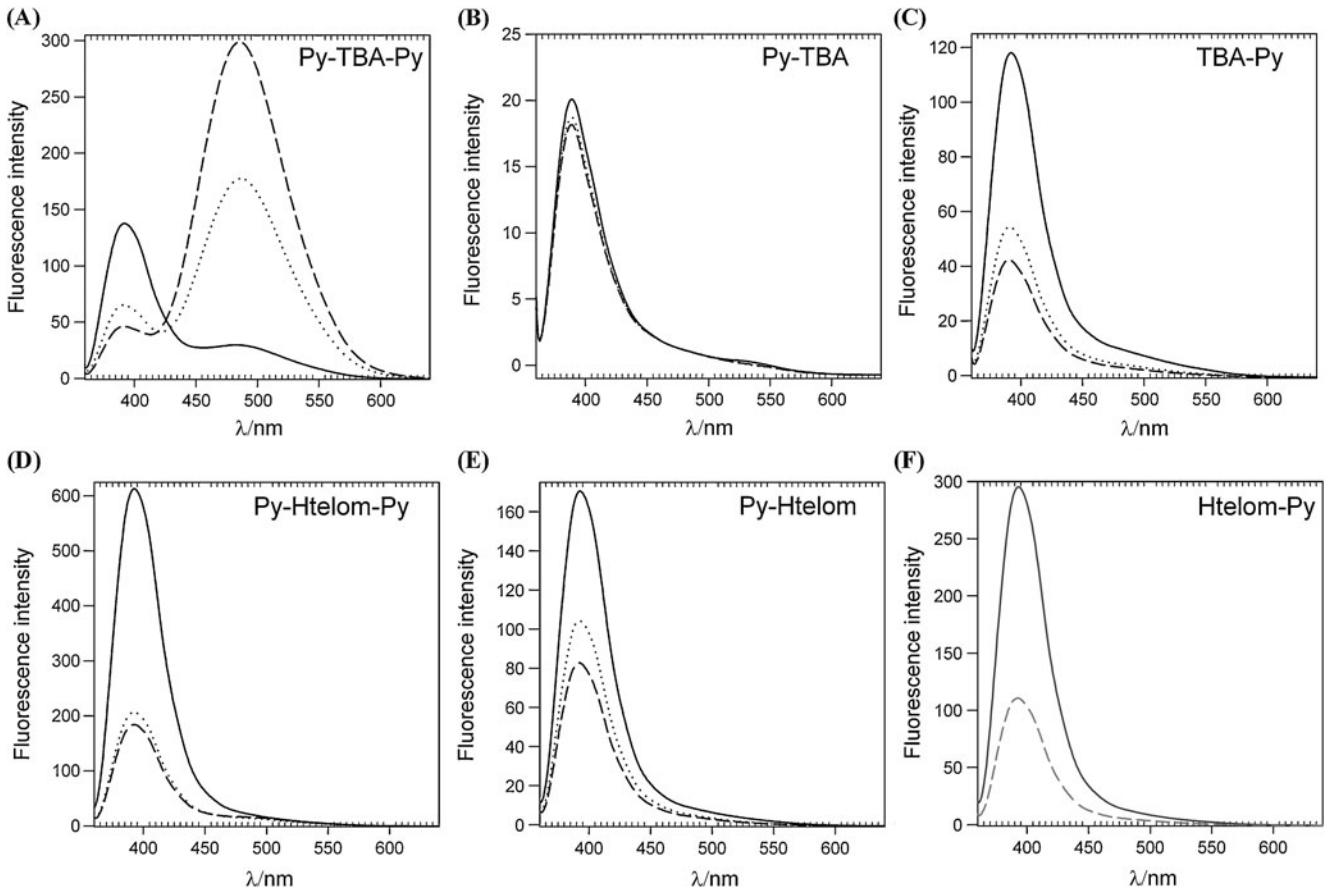

Fig. 1 The emission spectra of TBA (**a**, **b**, **c**) and Htelom (**d**, **e**, **f**) pyrene-labeled probes at varied K+ concentration: 0 mM KCl (*solid line*), 20 mM KCl (*dotted line*), 100 mM KCl (*dashed line*)

Table 2 Relative quantum yield (ϕ) and fraction of long wavelength emission (F>430 nm) of TBA and Htelom probes

Probe	KCl [mM]	ϕ	F>430nm
Py-TBA	No salt	0.008	0.12
	100 mM KCl	0.007	0.11
Py-TBA-Py	No salt	0.04	0.34
	100 mM KCl	0.12	0.91
TBA-Py	No salt	0.056	0.19
	100 mM KCl	0.019	0.16
Py-Htelom	No salt	0.077	0.16
	100 mM KCl	0.036	0.15
Py-Htelom-Py	No salt	0.13	0.13
	100 mM KCl	0.044	0.20
Htelom-Py	No salt	0.13	0.15
	100 mM KCl	0.048	0.14

was judged from the $\chi 2$ values ($\chi 2 \leq 1.5$) and random distribution of weighted residuals.

Results and discussion

Fluorescence spectrum of Py-TBA-Py is dominated by two components. A structured band in the region of 370–400 nm comes from the locally excited S1 state of pyrene and a broad fluorescence with the maximum around 480 nm is a result of pyrene sandwich—type excimer (Fig. 1a). At higher concentration of K^+ there is observed disappearing (quenching) of the monomer fluorescence at 390 nm as well as enhancement of excimer fluorescence. Such a behavior of Py-TBA-Py indicates formation of a G-quadruplex, which is stabilized by K^+ ions. As expected, TBA probes possessing pyrene moiety only at the one end of strand emits only monomer fluorescence (Fig. 1b and c). What is interesting, after adding K^+ into solution, there are very small changes in fluorescence intensity in case of Py-TBA labeled at the 5′ terminus (Fig. 1b). The fluorescence of free Py-TBA probe seems to be already quenched so efficiently that it is almost impossible to quench it further. On the contrary, TBA-Py, the probe having attached pyrene at the 3′ terminus, undergoes fluorescence quenching even in the presence of modest concentration of KCl, eg., 1 mM (Fig. 1c). The reason for the observed difference in spectral behavior between Py-TBA and TBA-Py is unclear, especially if one considers topology of formed G-quadruplexes. TBA prefers a chair-type architecture of quadruplex, where both strand termini are close to each other. Moreover, in both cases (5′ and 3′ labeled probes) linker between pyrene and oligonucleotide has the same length (a N-propylbutanoamide chain). Hence, the surrounding of

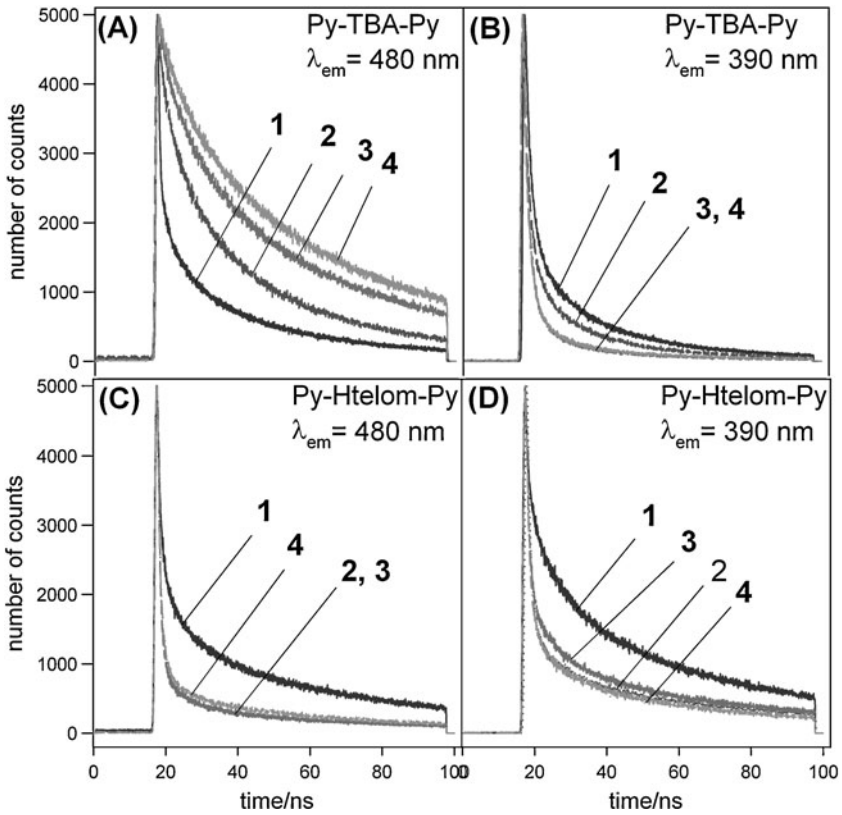

Fig. 2 Emission decay curves of Py-TBA-Py monitored at 480 nm (**a**) and 390 nm (**b**) as well as of Py-Htelom-Py monitored at 480 nm (**c**) and 390 nm (**d**). In each case probe (1 μM) was dissolved in 5 mM Tris-HCl buffer (pH 7.4) in the absence of metal cation (1) or in the presence of 1 mM KCl (2); 20 mM KCl (3) and 100 mM KCl (4). The excitation was at 340 nm

attached pyrene is supposed to be the same and no difference in fluorescence properties of these probes should be expected. Therefore, one can conclude that very tiny structural differences in pyrene microenvironment play crucial role in quenching efficiency of pyrene fluorescence by nucleobases.

The similar behavior has been observed for single-labeled Htelom probes (Fig. 1e and f). Free Py-Htelom probe emits 2-times weaker in comparison with Htelom-Py or Py-Htelom-Py notice that here the fluorescence is twice stronger than for Htelom-Py that may be explained by the presence of two pyrene tags. The explanation of spectral differences between Htelom probes is more straightforward. The Htelom sequence in the presence of potassium forms G-quadruplex with a hybrid-type structure [11]. The architecture of this quadruplex results in separation of both pyrenes and allows them to stack on the surfaces of external G-tetrads. However, there is no possibility that these moieties have the same surrounding (microenvironment). The pyrene moiety attached at the 5′ end can interact with two TTA loops (external and lateral), whereas pyrene linked at 3′ end of Htelom sequence could interact only with one lateral loop [17].

For all six probes, we also calculated contribution of long-wavelength fluorescence (λ_{em}>430 nm) and relative quantum yields of fluorescence, ϕ, with reference to ϕ of pyrene butanoic acid, assumed to be 1. The obtained results are collected in Table 2. At first glance it is visible a strong quenching of monomer fluorescence in case of TBA probes, especially for Py-TBA. Respective Htelom probes exhibit fluorescence at least 2,5 times stronger. It seems probable that 3D structure of Htelom probes gives pyrene labels more "freedom" and additionally, N-propylbutanoamide linker is sufficient to prevent from collision with loop, reducing static or electron transfer quenching [18–20]. The low fluorescence quantum yield of Py-Htelom in comparison to Htelom-Py confirms the hypothesis that pyrene attached at 5′ end is located close to two loops containing thymine bases, which can additionally quench pyrene fluorescence [18–20].

Table 3 Calculated lifetimes and its distribution in each decay

Probe	KCl Conc. [mM]	λ_{em}=390 nm				λ_{em}=480 nm			
		τ_1 (α_1)	τ_2 (α_2)	τ_3 (α_3)	<τ> [ns]	τ_1 (α_1)	τ_2 (α_2)	τ_3 (α_3)	<τ> [ns]
Py-TBA	no	<0.1 (0.99)	4.3 (<0.01)	35.0 (<0.01)	<1.0	n.d.	n.d.	n.d.	n.d.
	1	0.2 (0.96)	5.3 (0.03)	32.0 (0.01)	<1.0	n.d.	n.d.	n.d.	n.d.
	20	<0.1 (0.99)	6.5 (<0.01)	32.0 (<0.01)	<1.0	n.d.	n.d.	n.d.	n.d.
	100	0.2 (0.85)	5.1 (0.09)	29.0 (0.06)	<1.0	n.d.	n.d.	n.d.	n.d.
Py-TBA-Py	no	1.25 (0.71)	8.5 (0.17)	33.0 (0.12)	6.2	0.3 (0.79)	5.8 (0.11)	34.0 (0.10)	4.5
	1	1.1 (0.74)	7.0 (0.17)	29.0 (0.09)	4.5	0.5 (0.46)	7.4 (0.26)	35.0 (0.28)	12.4
	20	1.3 (0.84)	5.6 (0.13)	30.0 (0.03)	2.9	1.8 (0.16)	12.0 (0.33)	56.0 (0.51)	32.5
	100	1.2 (0.84)	6.2 (0.13)	33.0 (0.03)	3.0	5.8 (0.18)	27.0 (0.44)	84.0 (0.38)	45.1
TBA-Py	no	0.9 (0.52)	8.2 (0.23)	51.0 (0.24)	14.9	n.d.	n.d.	n.d.	n.d.
	1	1.0 (0.77)	5.0 (0.17)	36.0 (0.06)	3.6	n.d.	n.d.	n.d.	n.d.
	20	1.0 (0.73)	5.0 (0.22)	35.0 (0.05)	3.5	n.d.	n.d.	n.d.	n.d.
	100	1.0 (0.77)	5.3 (0.18)	36.0 (0.05)	3.7	n.d.	n.d.	n.d.	n.d.
Py-Htelom	no	0.5 (0.79)	7.9 (0.09)	66.0 (0.12)	9.4	0.5 (0.94)	6.3 (0.04)	51.0 (0.02)	1.8
	1	0.7 (0.90)	8.0 (0.06)	62.0 (0.04)	3.8	0.7 (0.87)	5.4 (0.11)	42.0 (0.02)	2.1
	20	0.6 (0.83)	7.7 (0.11)	47.0 (0.06)	4.1	0.7 (0.86)	6.4 (0.10)	34.0 (0.04)	2.7
	100	0.7 (0.87)	7.3 (0.08)	43.0 (0.04)	3.2	0.7 (0.86)	5.1 (0.10)	30.0 (0.04)	2.2
Py-Htelom-Py	no	0.4 (0.69)	7.8 (0.15)	59.0 (0.16)	10.9	0.6 (0.72)	6.4 (0.14)	59.0 (0.14)	9.3
	1	0.7 (0.83)	7.8 (0.09)	63.0 (0.08)	7.0	0.6 (0.89)	4.6 (0.08)	64.0 (0.03)	2.6
	20	0.6 (0.79)	6.9 (0.12)	63.1 (0.09)	7.0	0.6 (0.90)	4.9 (0.07)	62.0 (0.03)	2.6
	100	0.7 (0.81)	6.9 (0.10)	60.7 (0.09)	6.2	0.6 (0.87)	4.4 (0.08)	60.0 (0.05)	3.5
Htelom-Py	no	0.1 (0.85)	7.8 (0.03)	102.2 (0.12)	12.5	0.6 (0.82)	5.7 (0.10)	80.0 (0.08)	7.5
	1	0.7 (0.89)	7.2 (0.05)	68.0 (0.05)	5.0	0.8 (0.85)	5.6 (0.12)	52.0 (0.03)	3.0
	20	0.5 (0.91)	4.2 (0.06)	58.0 (0.03)	2.6	0.7 (0.89)	4.6(0.09)	41.0 (0.02)	2.0
	100	0.4 (0.90)	2.6 (0.09)	50.0 (0.01)	2.1	0.5 (0.89)	2.7(0.10)	38.0 (0.01)	1.1

The fluorescence quantum yield of free Py-Htelom-Py is similar to that of Htelom-Py. Taking into account that pyrene at 5′ terminus exibits twice weaker fluorescence than pyrene at 3′ terminus it indicates that quantum yield for Py-Htelom-Py is higher than calculated from contribution from both labels. What is interesting, the quantum yield of Py-Htelom-Py decreases at higher concentration of K^+, whereas fraction of long-wavelength emission increases slightly. To verify whether the fraction of long-wavelength emission for this probe comes from the traces of excimer fluorescence we have carried out lifetime emission study at different K^+ concentration. We also expected that these measurements could be helpful to clarify the source of difference in steady-state fluorescence properties between the 5′ and 3′ mono substituted probes.

The examples of the K^+ induced changes in fluorescence decay profiles at 480 nm (excimer fluorescence) and 390 nm (monomer fluorescence) for dual labeled Py-TBA-Py and Py-Htelom-Py probes are shown in Fig. 2. Only in case of Py-TBA-Py decay monitored at 480 nm one can observed a decrease in decay rate with potassium concentration (Fig. 2a). In all other cases potassium induced more efficient rate of deactivation of excited state of pyrene.

Time profiles for all samples were fitted by assuming a triexponential decay: $A + B_1\exp(-t/\tau_1) + B_2\exp(-t/\tau_2) + B_3\exp(-t/\tau_3)$, where B_i and τ_i mean the prexponential factor and lifetime of component i. The obtained results are collected in Table 3. We also calculated fractional contribution α_i of components corresponding to the particular lifetimes (Table 3). Similarly as in our previous studies [17] it was noticeable that more that 70% of the pyrene excited states in the double labeled oligonucleotides have an emission component with lifetime (τ_1) about 1 ns or even below (Table 3). The medium lifetime component (τ_2) is in the range of 4.3–8.5 ns (17–15%) for free probes, while long-lived species have lifetimes (τ_3) of 33 ns (12%) and 59 ns (16%) for TBA and Htelom—based probes, respectively. Figure 3 illustrates the changes in lifetimes for Py-TBA-Py probe upon increasing K^+ concentration in solution. As we have already indicated, Py-TBA-Py exhibits the linear dependence in excimer fluorescence with K^+ concentration [15]. Comparing lifetimes obtained for Py-TBA-Py at 390 nm with those at 480 nm, we observed the extension of τ_3 from ca. 30 ns to ca. 80 ns in the most concentrated KCl solution (100 mM). The fraction of long-lived species also increased almost 12 times from 3,3 to 38%. It confirms very strong excimer emission observed in

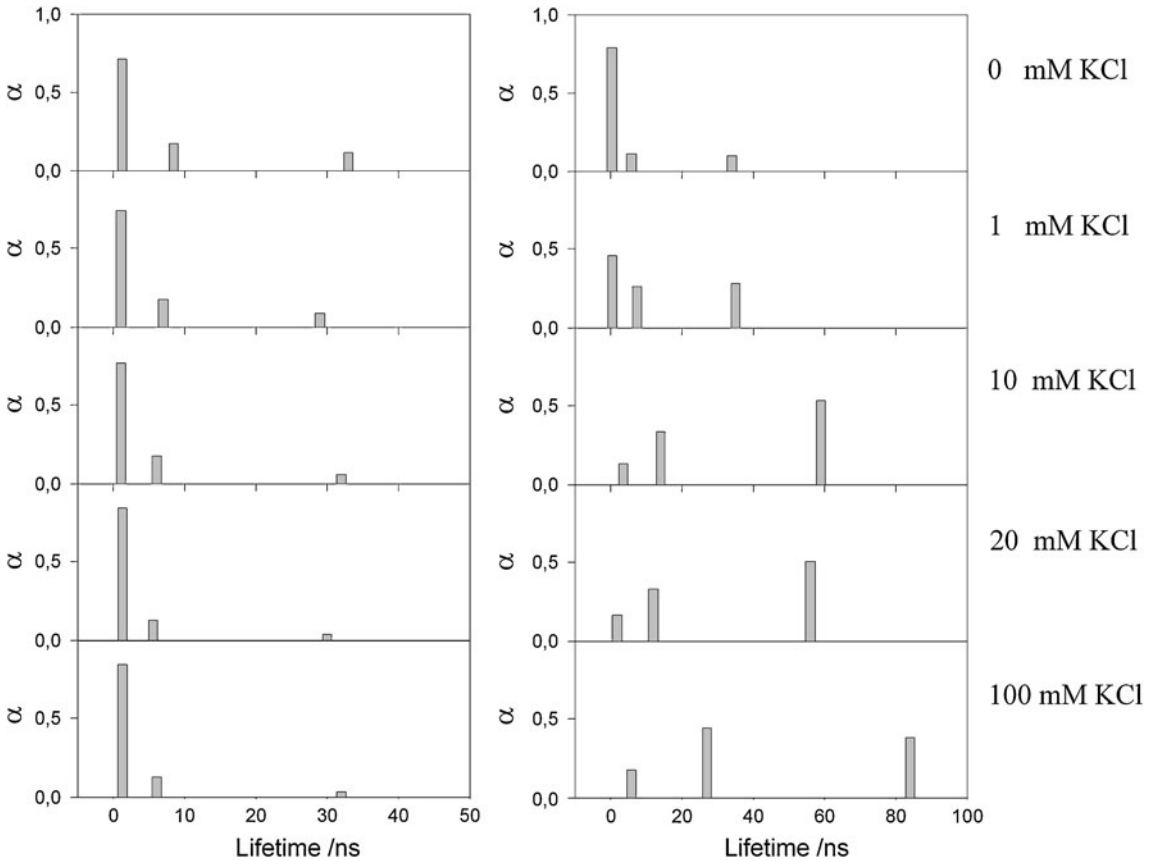

Fig. 3 Fractional distribution αi of emitting species of Py-TBA-Py obtained at 390 nm (*on the left*) and 480 nm (*on the right*) in the various K+ concentration

stead-state measurements. Similar tendency was observed by Marti at al., who calculated about 30% higher population of long-life species after forming excimer in their pyrene binary probe [21].

What is important in our case, the concentration of 1 mM KCl is enough to see redistribution of particular lifetimes (Fig. 3). We have not observed such kind a tendency in case of Py-Htelom-Py, which is consistent with the absence of strong long-wavelength emission for this probe. In this case the fractional contributions α_i as well as lifetimes calculated for 390 and 480 nm are very similar to each other, irrespectively of KCl contents. For example, τ_3 of Py-Htelom-Py, calculated at different concentrations of KCl, is in the range from 55 to 65 ns. This value (τ_3) is such long as measured for excimer Py-TBA-Py in 20 mM KCl but is still shorter than measured for PBA [17]. Therefore, one could speculate that the τ_3 lifetime and small increase in long-wavelength emission (Table 2) observed in fluorescence spectra of Py-Htelom-Py are an evidence of trace excimer formation. Such conclusion should be ruled out if one considers the hybrid-type structure of potassium quadruplex, which disturbs in face-to-face arrangement of pyrene rings. Moreover, K^+ induced excimer formation should result in gradual increase in τ_3 lifetime and/or α_3 fraction of excimer, which is not in the case for Py-Htelom-Py/K^+ system. An opposite effect is rather observed (Table 3), a τ_3 lifetime did not change, but fractional contribution (α_3) decreased with K^+ concentration. Finally, one can conclude, that Py-Htelom-Py does not exhibit excimer emission. Moreover, in case of all Htelom probes the fraction of long-wavelength emission seems to be dominated by a "tail" of monomer fluorescence. This is true also for Py-TBA and TBA-Py probes.

Therefore, in our discussion of decays for mono-labeled TBA and Htelom probes we focus on results calculated for 390 nm, which seem to be more reliable due to higher signal-to-noise ratio at this wavelength (Table 3). For each mono substituted probe, the fraction (α_3) of τ_3 has almost vanished in the presence of potassium, so these results correlate very well with relative quantum yields of fluorescence (ϕ) collected in Table 2. Interestingly, τ_3 calculated for free Py-TBA and TBA-Py at wavelength 390 nm are about twice shorter than those for free Py-Htelom and Htelom-Py, which is consistent with quantum yield results (Table 2). The τ_3 value for free Py-Htelom is very similar to τ_3 obtained for free Py-Htelom-Py. Surprisingly, τ_3 for free Htelom-Py is very long and amounts 102 ns. This lifetime is longer than value ~85 ns, which we calculated for 1-pyrenebutanoic acid (PBA) [17]. On the other hand, it is still shorter than lifetime 180 ns of for PBA in deaerated phosphate buffer reported by Zahavy et al. [22]. The essential difference, which we noticed after addition of K^+ ion, is the gradual shortening of τ_3 for mono substituted Htelom probes contrary to Py-Htelom-Py, for which values of lifetimes were relatively fixed but their redistribution (α_i changed) was induced by potassium addition.

The average lifetime, $<\tau>$ defined as $\alpha_1\tau_1 + \alpha_2\tau_2 + \alpha_3\tau_3$, calculated for all six probes, show the strong linear increase upon addition of K^+ ions only in case of Py-TBA-Py (Table 3).

Summarizing, we carried out the direct measurements of K^+-induced changes in steady-state fluorescence spectra as well as in decay profiles of six G-quadruplex probes. Excimer emission of pyrene dominated only in case of Py-TBA-Py probe carrying pyrene moieties at both ends. On the contrary, analogous Htelom-based probe, Py-Htelom-Py, exhibited negligible long-wavelength emission and shorter average lifetime. In all cases after attachment to oligonucleotide, the pyrene fluorescence was strongly quenched in comparison with free pyrene label (PBA) and we believe that additional study involving nanosecond time-resolved laser flash photolysis experiments would put the light upon the mechanism of observed fluorescence quenching [23].

Our results confirmed that the differences in fluorescent properties of Htelom and TBA probes labeled with pyrene are connected with tertiary structure of these conjugates.

Conclusions

In conclusion, we showed quantitatively that the potassium affects lifetimes of TBA and Htelom G-quadruplexes double-labeled with pyrene. Moreover, TBA as well as Htelom probes possessing pyrene at 3′ end are also gradually quenched by potassium ion. These results indicate that even single-labeled pyrene probes are an alternative to be used in bioanalytical applications.

However, the linear dependence of the average lifetime of Py-TBA-Py upon K^+ addition is unique. These results, once more indicate, that the chair-type G-quadruplexes are good scaffold to construct potassium-sensitive sensors. Especially we recommend to label such sensors with pyrene which excimer fluorescence could be also exploited, for example, in time-resolved fluorescence imaging microscopy for biological applications [24].

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