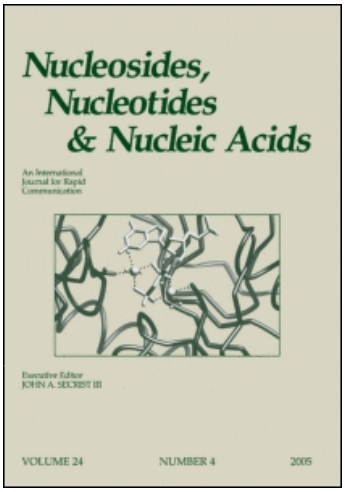


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

5'-BIS-PYRENYLATED OLIGONUCLEOTIDES DISPLAY ENHANCED EXCIMER FLUORESCENCE UPON HYBRIDIZATION WITH DNA AND RNA

E. Kostenko[a]; M. Dobrikov[a]; N. Komarova[a]; D. Pyshniy[a]; V. Vlassov[a]; M. Zenkova[a]
[a] Novosibirsk Institute of Bioorganic Chemistry, Novosibirsk, Russia

Online publication date: 31 December 2001

To cite this Article Kostenko, E. , Dobrikov, M. , Komarova, N. , Pyshniy, D. , Vlassov, V. and Zenkova, M.(2001) '5'-BIS-PYRENYLATED OLIGONUCLEOTIDES DISPLAY ENHANCED EXCIMER FLUORESCENCE UPON HYBRIDIZATION WITH DNA AND RNA', Nucleosides, Nucleotides and Nucleic Acids, 20: 10, 1859 — 1870
To link to this Article: DOI: 10.1081/NCN-100107197
URL: http://dx.doi.org/10.1081/NCN-100107197

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(10&11), 1859–1870 (2001)

5′-BIS-PYRENYLATED OLIGONUCLEOTIDES DISPLAY ENHANCED EXCIMER FLUORESCENCE UPON HYBRIDIZATION WITH DNA AND RNA

E. Kostenko, M. Dobrikov, N. Komarova, D. Pyshniy, V. Vlassov, and M. Zenkova*

Novosibirsk Institute of Bioorganic Chemistry, 8 Lavrentiev ave., 630090, Novosibirsk, Russia

ABSTRACT

A simple one-step procedure was applied for synthesis of oligonucleotide conjugates bearing two pyrene residues at the 5′-phosphate of oligonucleotide. Excimer fluorescence intensity of the conjugates is highly sensitive to duplex formation: binding of the bis-pyrenylated oligonucleotides to their DNA and RNA targets leads 10-fold increase of fluorescence. The data show that excimer fluorescence intensity of the conjugates depends linearly on the concentration of target DNA and permits quantification of DNA in solution.

INTRODUCTION

Conjugates of oligonucleotides with fluorescent compounds find broad applications as probes for detection of specific nucleic acids sequences in wide range of assays, including diagnostic of genetic disorders and infections of various etiologies[1,2]. In recent years considerable effort has been expended in designing of oligonucleotide probes which displays considerable changes in the emission spectrum upon duplex formation with target RNA or DNA

*Corresponding author. Fax: +7-383-2-33-36-77; E-mail: marzen@niboch.nsc.ru.

since this type of conjugates can be used for solution based assays[3–5] or investigation of RNA structure[6–8]. No separation step in needed for the detection, which improves the overall rate of the assay.

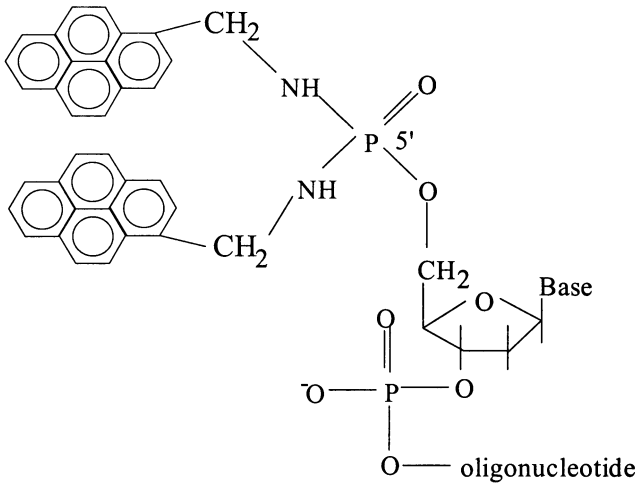
Pyrene is one of the fluorescent dyes used for development of sensitive probes for detection of DNA[9–12] and RNA[6,8,13–15]. Rather long fluorescence lifetime (up to 400 ns) makes it sensitive to the presence of quenchers[16]. The fluorescence of a single pyrene residue linked to an oligonucleotide was shown to be affected by duplex formation[6,8,9–12,14]. Pyrene molecules can form complex called excimer, consisting of one ground state and one excited state species, which fluoresce in the visible region[16]. Because of spatial requirements, excimer formation is very sensitive to various interactions involving the label, which makes pyrene excimer fluorescence very attractive tool for investigation of nucleic acids interactions and structures. Recently, several reports on excimer-forming pyrene containing probes proved the possibility of successful use of pyrene excimer fluorescence for nucleic acids assays. Authors developed different strategies of excimer formation. Excimer fluorescence of the pair of 5′-end and 3′-end pyrene labeled oligonucleotide, complementary to adjacent region within target DNA was successfully applied for detection of point mutation and quantification of DNA[17–19,23]. Oligonucleotide conjugates, exhibiting excimer fluorescence due to presence of two[20,21,23] or multiple pyrene residues in oligonucleotide conjugate[24] were developed. Some attempts to develop oligonucleotide probe, displaying excimer fluorescence have been complicated by multi-step synthesis of conjugates[21,24]. In some cases oligonucleotides, bearing multiple pyrene residues were not sensitive enough to hybridization with the target sequence[24].

In present work we report simple one-step synthesis of oligonucleotide conjugates displaying excimer fluorescence in which two pyrenyl-1-methylamine residues are attached to the 5′-phosphate of oligonucleotides. The conjugates exhibit enhancement of excimer fluorescence intensities upon duplex formation with complementary RNA and DNA in target concentration dependent manner.

EXPERIMENTAL

Oligonucleotides

Oligodeoxyribonucleotides (**Chart** 1) were synthesised on ASM-102U synthesiser (BIOSSET, Novosibirsk, Russia) using standard phosphoramidite chemistry. DNA oligomers were purified by consecutive ion-exchange and reverse-phase HPLC. Oligoribonucleotide **rTg-I** was provided by TriLink Laboratories Inc., San-Diego. The oligonucleotides were homogeneous as assayed by 15% PAGE in denaturing conditions followed by staining with Stains-All.



dTg-I	**$^{5'}$(dCCAAGGAGCGCGAGGTCG)$^{3'}$**
rTg-I	**$^{5'}$(rCCAAGGAGCGCGAGGUCG)$^{3'}$**
dTg-II	**$^{5'}$(dTGAATCTGGAGGAAGACA)$^{3'}$**
dTg-III	**$^{5'}$(dCATCCATGGGGCTCCACTT)$^{3'}$**
I	**$^{5'}$(dGACCTCGCGCTCCTTG)$^{3'}$**
II	**$^{5'}$(dGTCTTCCTCCAGATTC)$^{3'}$**

Designation **I** and **II** are used for unmodified oligonucleotides. **I**^{bis} and **II**^{bis} indicate bis-pyrenylated oligonucleotides. Structure of bis-pyrenylated oligonucleotide are shown on the top of this **CHART**. Regions of target RNA and DNAs complementary to oligonucleotides are underlined.

Chart 1.

Synthesis of bis-Pyrenylated Oligonucleotide Conjugates

Two pyrene residues were attached to the 5′-terminal phosphate of the oligonucleotides that had been activated with a mixture of triphenylphosphine (Ph_3P) and 2,2′-dipyridyldisulfide $(PyS)_2$ in the presence of 4-(N,N-dimethylamino)pyridine (DMAP)[25,26]. Briefly, 1–10 A_{260} O.D. (5–100 nmol) of oligonucleotide in water (concentration 100 A_{260}/ml) was precipitated with N-cetyl-N,N,N-trimethylammonium bromide, centrifuged, and the

oligonucleotide pellet was thoroughly dried in vacuum over P_2O_5. To activate phosphate the reaction mixture containing 5–10 nmol of the dried N-cetyl-N,N,N-trimethylammonium salt of the oligonucleotide, 0.05 ml of dried dimethylsulfoxide (DMSO), 10 mg (38 μmol) of Ph_3P, 10 mg (50 μmol) of $(PyS)_2$, and 10 mg (80 μmol) of DMAP, was subjected to several (3–5) brief (1 min) heatings at 50 °C followed by intensive vortexing and incubation at 25 °C for 10 min. Then 2 mg of pyrenyl-1-methylamine hydrochloride was added and the reaction was performed for 40 min at 25 °C at constant vortexing. The reaction was quenched by precipitation of the oligonucleotide conjugate with 1.5 ml of 2% lithium perchlorate solution in dry acetone. Purification of the bis-pyrenylated oligonucleotide was performed by reversed-phase HPLC using LiChrosorb RP-18, 10 μm (Merck), 4.6×250 mm column, Waters 600E chromatograph and Waters 484 tunable absorbance detector (USA). Linear (0 to 30%) gradient of acetonitrile in 0.05 M $LiClO_4$ pH 7.5, (flow rate 2 ml/min) was used. Lithium salt of bis-pyrenylated oligonucleotide (5–100 nmol) was precipitated with acetone, centrifuged, dried, and dissolved in water. Bis-pyrenylated oligonucleotide conjugates were stable under storage in water at –20 °C for several months. The conjugates were characterized by polyacrylamide gel electrophoresis, reverse-phase HPLC, UV- and fluorescence spectroscopy, and by liquid chromatography – mass-spectrometry. LC-MS analysis has been done by Dr. Hans Gaus (ISIS Pharmaceutical): for conjugate $\mathbf{I}^{\mathbf{bis}}$: expected 5001.13, found 4999,8 for the peak with retention time 21.13–22.71; charge states −3 and −4. UV and fluorescent spectra of the conjugates are shown in Fig. 1a and Fig. 1b, respectively.

Hybridisation of bis-Pyrenylated Oligonucleotides to Short Complementary RNA and DNA Targets

Binding of bis-pyrenylated oligonucleotide conjugates to the RNA and DNA targets was investigated at 37 °C in standard hybridisation buffer containing 50 mM HEPES pH 7.5, 5 mM $MgCl_2$, 200 mM KCl, 0.5 mM EDTA. Solution of bis-pyrenylated oligonucleotide conjugate (5×10^{-7} M) was titrated with increasing concentration of RNA or DNA targets at concentration ranging from 5×10^{-9} M to 1×10^{-6} M. Reaction mixtures were allowed to set under hybridization conditions until no fluorescence changes were detected before fluorescence measurements were performed.

Fluorescence Measurements

Fluorescence spectra were recorded on a MPF4 Hitachi spectrofluorimeter using a bandwidth of 15 nm and 0.5×2 cm quarts cuvette with

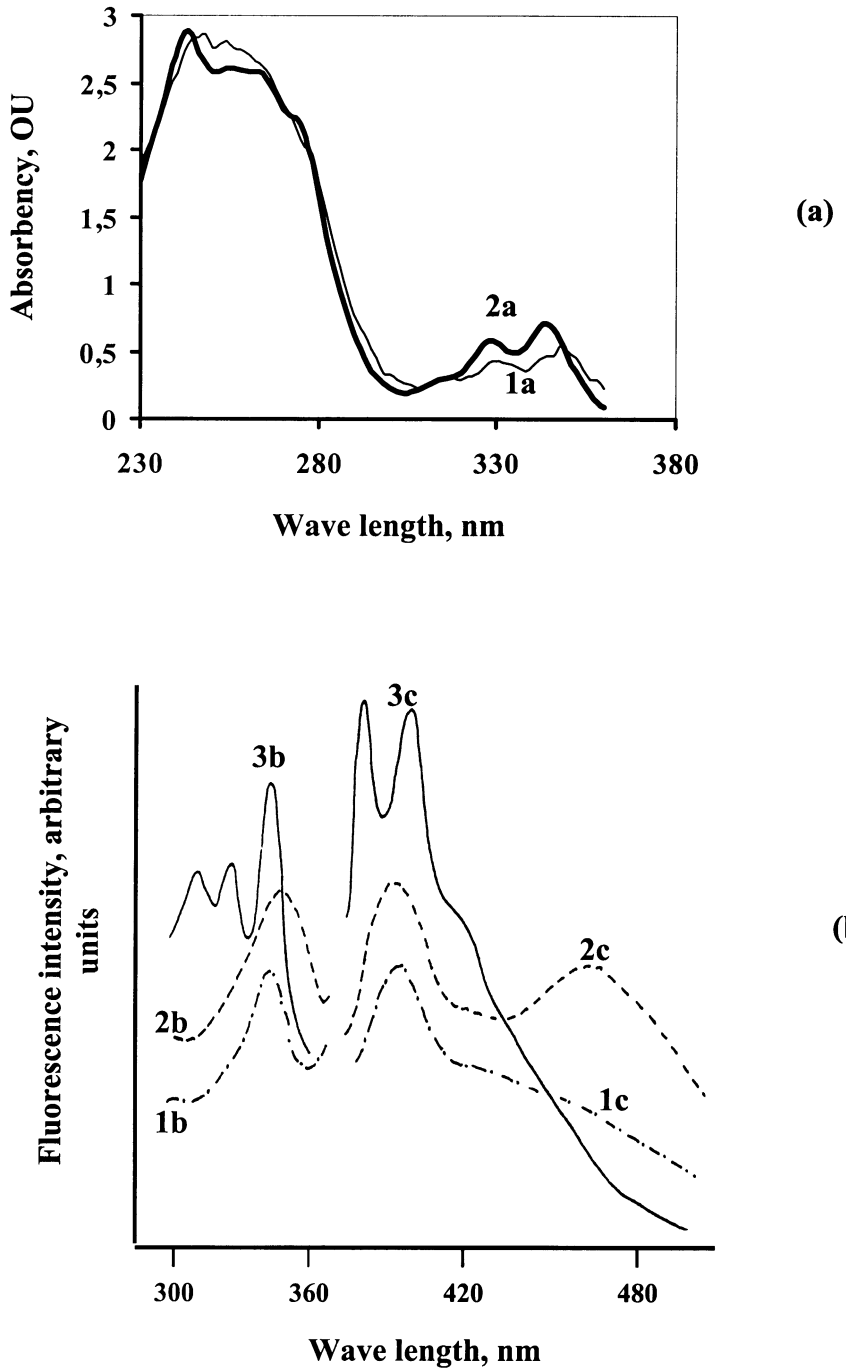


Figure 1. UV-absorbance (a), fluorescence excitation (b) and emission (c) spectra of mono-pyrenylated (1) or bis-pyrenylated oligonucleotide I (2) and pyrenyl-1-methylamine (3). Concentration of pyrenyl 1-methyl amine in ethanol was 5×10^{-8} M. Oligonucleotide conjugate concentrations were 5×10^{-7} M in 50 mM HEPES-KOH, pH 7.5; containing 200 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA at 37 °C.

light pass 1 cm. The cell holder was thermostated by circulating water controlled with LKB-2219 Multitemp II Thermostatic Circulator. Pyrene was excited at 343 nm. Pyrene fluorescence emission was monitored at 382 nm for monomer and 476 nm for excimer fluorescence. Emission spectra were measured using reference dye (rodamine-B) to compensate for lamp fluctuation. In all spectra the background emission from the buffer alone was subtracted.

Thermal Denaturation Experiments

Thermal denaturation of oligonucleotide complexes was performed in 50 mM cacodylate buffer pH 7.5, containing 5 mM $MgCl_2$, 200 mM KCl, 0.5 mM EDTA. The concentration of each component was 1×10^{-5} M. The melting curves were registered in multiwave length mode, at least 3 different wavelength were used. Absorption was measured for each oligonucleotide mixture as a function of temperature, heating rate was 0.5–1 °C/min in the UV detector Millichrom liquid chromatograph in a thermoregulated sapphire cell ($V = 2\,\mu l$) specially designed for this purpose. The equilibrium optical melting curves were found on the basis of more than 600 experimental points with the step 10 points/°C and were completely reversible in heating-cooling processes. The first derivatives of optical melting curves versus temperature were calculated using a gradient of linear approximation by 10 experimental points. Tm of the duplex was the average temperature corresponding to the maximum of first derivatives of optical melting curve of duplexes formed by stechiometrical mixture of DNA or RNA target and the probe.

RESULTS AND DISCUSSION

Synthesis of pyrenyl-1-methylamine was described earlier[27]. Oligonucleotides **I** and **II** were used for synthesis of bis-pyrenylated conjugates $\mathbf{I}^{\mathbf{bis}}$ – $\mathbf{II}^{\mathbf{bis}}$ (**Chat 1**). To synthesize 5′-bis-pyrenylated oligonucleotide conjugates, 5′-phosphate of the oligonucleotides was activated with dipyridyl-disulfide (Py_2S_2), triphenyl-phosphine (PPh_3) and dimethylaminopyridine (DMAP) in dry DMSO and then treated with 1-pyrenyl-methylamine. Similar protocol was employed for oligonucleotide derivatisation with N-methyl-N-(2-chloroethyl)-benzylamine[28] and pyrenyl-1-methylamine[29] to yield alkylating oligonucleotide derivative or mono-fluorophore labeled oligonucleotides.

The two minor modification of the described protocols[25,26] were the following. We used increased concentration of activating agents (0.4 M PPh_3, 0,5 M Py_2S_2, and 0.8 M DMAP) and fluorophore (0.08 M pyrenyl-1-methylamine) and during the activation step, reaction mixtures were several

times briefly (1 min) heated at 50 °C. This synthetic procedure provides bis-pyrenylated oligonucleotides with 82–98% yields, mono-fluorophore labeled oligonucleotide is formed as by-product. Previously, it was shown that di-anilide of pT(Ac) is formed in the reaction of pT(Ac) with Py_2S_2 and PPh_3 in the presence of aniline[26]. Recently, the attachment of two alkylamine residues to the 5′-phosphate of oligonucleotides in the above conditions has been proven by NMR studies[30].

UV spectra of the conjugates (Fig. 1a) demonstrate the presence of two pyrene residues in their structure: they have two absorption maxima at 260 and 342 nm and absorption intensity at 342 nm is enhanced twice for bis-pyrenylated oligonucleotide conjugate as compared to the mono-pyrenylated oligonucleotides. In the fluorescence emission spectra of the oligonucleotide conjugates $\mathbf{I}^{\mathbf{bis}}$ (Fig. 1b), the maxima at 382 nm and 476 nm correspond to pyrene monomer and excimer fluorescence, which is also consistent with the proposed structure with two pyrene residues.

Fluorescence emission at 384 nm and 476 nm and fluorescence quantum yields were assigned for the free conjugates and for complexes of the conjugates $\mathbf{I}^{\mathbf{bis}}$ and $\mathbf{II}^{\mathbf{bis}}$ with complementary DNA and RNA targets (Table 1). Conjugation of pyrene residues to oligonucleotides causes a strong quenching of pyrene monomer fluorescence and appearance of excimer fluorescence. 46 and 26-fold decrease in quantum yield of monomer fluorescence at 382 nm was observed for conjugates $\mathbf{I}^{\mathbf{bis}}$ and $\mathbf{II}^{\mathbf{bis}}$, respectively, as compared to pyrenyl-1-methylamine. Probably, in the intramolecular structure of the conjugates, one of the pyrene residues is involved in stacking interactions with adjacent nucleobases of oligonucleotide, which interferes with excimer formation. Excimer fluorescence of the conjugates is sensitive to their interactions with the complementary strands: the conjugates $\mathbf{I}^{\mathbf{bis}}$ and $\mathbf{II}^{\mathbf{bis}}$ exhibit 8, 10, and 9 fold increase in the excimer fluorescence intensities upon binding with corresponding targets (**dTg-I**, **rTg-I**, and **dTg-II**, respectively) (Table 1, Fig. 2). Enhanced excimer fluorescence of the conjugates in the duplexes suggests that hybridization releases the nucleobase stacked pyrene residue. Perhaps, the short linker between pyrene residue and oligonucleotide 5′-phosphate provides some steric constrains and prevents intercalation of the pyrene residue in rigid duplexes, although in the flexible single-stranded $\mathbf{I}^{\mathbf{bis}}$ and $\mathbf{II}^{\mathbf{bis}}$ interaction of the fluorescent group with nucleobases can occur. Upon duplex formation, these interactions are disrupted and pyrene excimer is restored. Thus, enhancement of excimer fluorescence upon hybridization indicates that pyrene excimer is formed upon conjugate binding with complementary strand. There is a possibility that bis-pyrenylated oligonucleotides possess their own structure with one or two pyrene residues involved which affects the duplexes stability. Tm measurements show (Table 1) that stabilities of duplexes formed by the conjugates with corresponding targets are slightly affected by the presence of pyrene residues in the conjugates (ΔTm are +2 and +3 °C for duplexes formed by $\mathbf{I}^{\mathbf{bis}}$ and $\mathbf{II}^{\mathbf{bis}}$, respectively).

Table 1. Emission Spectra Data (λ_f), Monomer Fluorescence Quantum Yields (Φ_f), Excimer/Monomer Ratios (I_{ex}/I_m), Melting Temperature (Tm) Values for bis-Pyrenylated Oligonucleotides $\mathbf{I}^{bis}$ and $\mathbf{II}^{bis}$ and Their Duplexes with Target DNAs and RNA

Oligomers[1,2]	λ_f	Φ_f[3]	I_{ex}/I_m	$I_{ex}ds/I_{ex}-ss$[5]	Tm, °C[4]
Pyrenyl-1-methylamine	376	− 0,6			
$\mathbf{I}^{bis}$	384, 476	0,013	1.04		62°
$\mathbf{I}^{bis}$+**dTg-I**	384, 476	0,058	2,5	8	75°
$\mathbf{I}^{bis}$+**rTg-I**	384, 476	0.059	3.4	10	76°
$\mathbf{II}^{bis}$	384, 476	0,023	1,2		45°
$\mathbf{II}^{bis}$+**dTg-II**	384, 476	0,074	2	9	65°
I					39°
I+**dTg-I**					73°
II					21°
II+**dTg-II**					62°

[1]Oligonucleotide conjugates (5×10^{-7} M) were dissolved in 50 mM HEPES, pH 7.5; 5 mM $MgCl_2$, 0.2 M KCl, 0.1 mM EDTA at 37 °C. Pyrenyl-1-methylamine (5×10^{-7} M) was dissolved in ethanol. Duplexes were prepared as follows: 5×10^{-7} M solution of oligonucleotide conjugate was titrated with corresponding targets in the same buffer at 37 °C.

[2]Concentrations of targets and unlabeled oligonucleotides were determined from absorbance at 260 nm, based on their calculated extinction coefficients [31]. Contribution of pyrene moiety to the absorbance at 260 nm of the oligonucleotide conjugate was the same as for pyrenyl-1-methylamine ($\varepsilon_{260}=25{,}000\ M^{-1}\,cm^{-1}$) [29].

[3]Monomer fluorescence quantum yields (Φ_f) of free pyrenyl-1-methylamine and of the pyrene labeled oligonucleotides were determined by comparing the integral fluorescence spectra of samples with those of the spectrum of standard solution of pyrenebutanoic acid [32]. The Φ_f of pyrenyl-1-methylamine was measured to be the same as for pyrenebutanoic acid.

[4]Tm's of the duplexes and intramolecular structures of the conjugates were determined as described in experimental.

[5]Ratio of excimer (I_{ex}) fluorescence intensities of target bound and free bis-pyrenylated oligonucleotide conjugate. I_m-pyrene monomer fluorescence intensity.

On the other hand, a considerable increase of Tm's of the intramolecular structures of the conjugates is observed: ΔTm for conjugates $\mathbf{I}^{bis}$ and $\mathbf{II}^{bis}$ are +23 and +24 °C compare to non-modified oligonucleotides **I** and **II**, respectively. Apparently the pyrene residues are involved in formation of some relatively stable intramolecular structures with oligonucleotide part of the conjugates. Indeed, no changes of Tm values were observed with increasing or decreasing of concentrations of the conjugates which is characteristic for intramolecular structure formation.

Considerable difference in excimer fluorescence intensity between free and target bound bis-pyrenylated oligonucleotides provides a possibility for application of these conjugates for real-time detection of DNA or RNA in solution. To test the conjugates in solution hybridization assay, the bis-pyrenylated oligomers $\mathbf{I}^{bis}$ and $\mathbf{II}^{bis}$ were titrated with solutions of the corresponding DNA and RNA targets. The excimer fluorescence intensity of

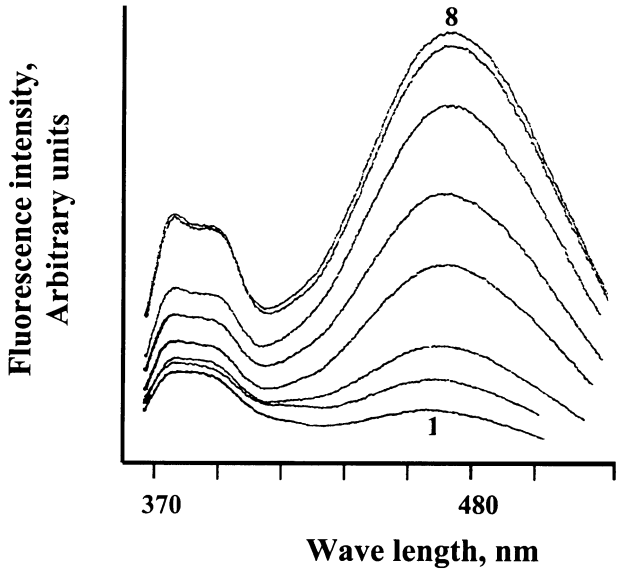


Figure 2. Fluorescence monitoring titration of 5′-bis-pyrenylated oligonucleotide $\mathbf{I}^{bis}$ with complementary DNA target in 50 mM HEPES-KOH, pH 7.5; containing 200 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA at 37 °C. **(1)** Fluorescence emission spectra of $\mathbf{I}^{bis}$ (5×10^{-7} M). (**2–8**) Fluorescence emission spectra of $\mathbf{I}^{bis}$ (5×10^{-7} M) in the presence of increasing concentrations of **dTg-I**. For spectra 2–8, **dTg-I** concentrations were 10^{-7} M, 2×10^{-7} M, 5×10^{-7} M, 7×10^{-7} M, 1×10^{-6} M, 2×10^{-6} M, 3×10^{-6} M, respectively.

$\mathbf{II}^{bis}$ increases with increasing of **dTg-II** concentration (Fig. 2). Incubation of non-complementary DNA (**dTg-III, Chat 1**) with conjugates $\mathbf{I}^{bis}$ and $\mathbf{II}^{bis}$ results in neither hypochromicity nor changes in fluorescence spectra of the conjugates. Thus the increase in excimer fluorescence observed upon addition of complementary target to bis-pyrenylated oligonucleotides is the consequence of duplex formation.

To enhance the sensitivity of the assay, hybridization could be carried out in solution of chromophore which absorbs light at the wavelength close to monomer emission maximum and does not absorb the excimer emission of the bis-pyrenylated oligomers. Various chromophores which may be employed as quenchers include aromatic aldehydes, ketones and nitro-compounds. An example of the fluorescence titration assay of $\mathbf{I}^{bis}$ with **dTg-I** in the presence of nitrobenzoic acid as a quencher of monomer fluorescence is shown in Fig. 3. Excimer fluorescence of conjugate $\mathbf{I}^{bis}$ was measured as function of **dTg-I** concentration. The data show that excimer fluorescence intensity of the bis-pyrenylated oligomer depends linearly on the amount of target DNA in the concentration range from 10^{-6} to 10^{-9} M and permits quantitative determination of target DNA in the solution.

Thus the designed pyrene labeled oligonucleotide conjugates exhibit strong enhancement of excimer fluorescence upon duplex formation. Short

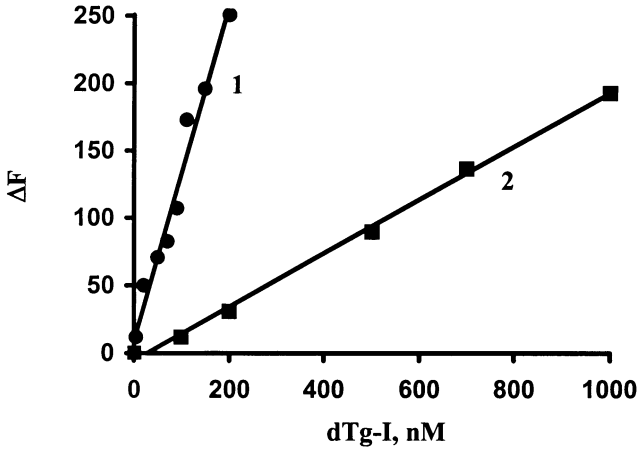


Figure 3. Increase of the excimer fluorescence of conjugate $\mathbf{I}^{\mathbf{bis}}$ upon duplex formation with target **dTg-I** in 50 mM HEPES-KOH, pH 7.5; containing 200 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA at 37 °C. λ_{ex} is 343 nm; λ_{em} is 476 nm. Curve 1 – titration of 2×10^{-7} conjugate $\mathbf{I}^{\mathbf{bis}}$ M with target DNA at concentration 5×10^{-9} M, 2×10^{-8}, 5×10^{-8}, 7×10^{-8}, 9×10^{-8}, $1{,}1 \times 10^{-7}$ M, $1{,}5 \times 10^{-7}$ M, 2×10^{-7} M in the presence of nitrobenzoic acid (2×10^{-5} M). Curve 2 – titration of 5×10^{-7} M conjugate $\mathbf{I}^{\mathbf{bis}}$ with target DNA at concentration 1×10^{-7} M, 2×10^{-7}, 5×10^{-7}, 1×10^{-6} M. The measurements were performed on spectrofluorimeter Hitachi MPF-40; measurements for curve 1 were made at 10 times higher sample sensitivity than for curve 2.

spacer prevents intercalation of pyrene residues into duplex. These oligonucleotide conjugates could find application for quantification on target DNA or RNA in solution or for investigation of nucleic acid structures. Simple synthetic procedure used in this study could facilitate further application of bis-pyrenylated oligonucleotides in many researches and applied techniques.

ACKNOWLEDGMENTS

We thank Dr. M. Manoharan for helpful discussions and Dr. H. Gaus for LC-MS analysis of bis-pyrenylated oligonucleotide conjugates. We acknowledge Dr. A. Lebedev and TriLink BioTechnologies for synthesis **rTg-I**. This work was supported by research grant from INTAS (INTAS-RFBR 95-0653), from Russian Foundation of Basic Research (RFBR 99-04-49559), and Interdisciplinary grant N 26. from Siberian Branch of Russian Academy of Science.

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Received March 5, 2001
Accepted July 2, 2001