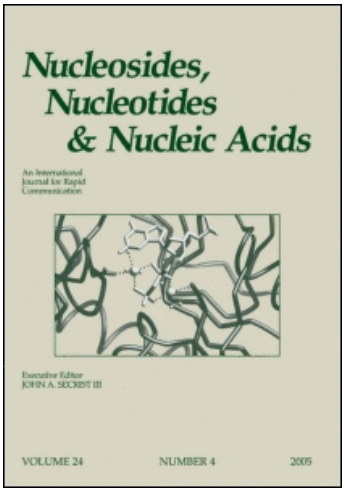

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Fluorescent-Labeled Oligonucleotide Probes with Non-Nucleotide Linker: Detection of Hybrid Formation by Fluorescence Anisotropy

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To cite this Article Patil, Sucheta V. and Salunkhe, Manikrao M.(1996) 'Fluorescent-Labeled Oligonucleotide Probes with Non-Nucleotide Linker: Detection of Hybrid Formation by Fluorescence Anisotropy', Nucleosides, Nucleotides and Nucleic Acids, 15: 10, 1603 — 1610
To link to this Article: DOI: 10.1080/07328319608002460
URL: http://dx.doi.org/10.1080/07328319608002460

NUCLEOSIDES & NUCLEOTIDES, 15(10), 1603-1610 (1996)

FLUORESCENT-LABELED OLIGONUCLEOTIDE PROBES WITH NON-NUCLEOTIDE LINKER : DETECTION OF HYBRID FORMATION BY FLUORESCENCE ANISOTROPY

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ABSTRACT *: Fluorescein labeled oligonucleotide probes with non-nucleotide linker have been synthesized and used to monitor hybrid formation to detect DNA sequences in solution. Fluorescence anisotropy, r, was adopted as an index to monitor triple helix formation and the behaviour of F-Probe in solution. An appreciable increase in anisotropy was observed upon use of non-nucleotide linker in the fluorescence probe as compared to that of the F-Probe without non-nucleotide linker.*

INTRODUCTION

During the last two decades the use of nonradioactive markers instead of radioisotopes has gained increasing importance in bioanalysis[1]. DNA-Probe method has not widely spread amongst researchers as it involves the use of radioisotopes. Major disadvantages of isotope use such as rapid decay, radiation exposure and the need of specific isotope facilities have been overcome by the development of nonradioactive labeling and detection techniques[2-5].

Fluorescein-labeled oligonucleotides, F-Probes, were utilized as the labeled-DNA-probes and the fluorescence anisotropy, 'r', was measured in solution containing oligonucleotides[6]. The duplex formation between F-Probe and a single stranded DNA has been monitored by the change in 'r' values.

In our report here, fluorescent-labeled oligonucleotides with a non-nucleotide linker were utilized as labeled-DNA-probes

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and the fluorescence anisotropy, 'r', was measured in solution containing oligonucleotides. The triple helix formation between the oligonucleotide probe with a non-nucleotide linker and a single stranded DNA has been monitored by effective change in 'r' values as compared to that of F-Probe without non-nucleotide linker.

RESULTS AND DISCUSSION

The reversible association of an oligonucleotide with a complementary sequence is of basic importance in biotechnology. Studies of modified oligonucleotides have been conducted with the objective of gaining a further understanding and control of hybridization[7-10].

Oligonucleotide derivatives in which the linker X = $-P(O)(O^-)O(CH_2)_5NHCOC_6H_4CONH(CH_2)_6OP(O)(O^-)-$ is present, can serve as a bridge between the 3' terminal oxygen atom of one oligomer and the 5' terminal oxygen atom of another[11] one. This linker serves as a replacement either for the single stranded oligonucleotide loop characteristic of a hairpin structure or for an oligonucleotide segment connecting two pyrimidine oligonucleotide tracts of a triple helix. Two oligo (dT) strands with this non-nucleotide linker bind selectively to the oligo (dA) target.

A striking feature of the results of the present study is the stability of the complexes that are formed. Interaction of $dT_{||}$(1eq.) with $dA_{||}$(1eq.) (Tm 22°C) and also $dT_{||}$(2eq.) with $dA_{||}$ (1eq.) (Tm 25°C) is weak in sharp contrast, the linked oligomer $dT_{||}X\ T_{||}$ forms stable complex with $dA_{||}$ (Tm 54°C) under the same conditions. Melting temperature (Tm) values for complexes derived from the linked probes are much higher than for Watson-Crick type duplexes containing the same base pairs. Presumably, Hoogsteen as well as Watson-Crick hydrogen bonding stabilizes a bimolecular 'triplex'.

Taking into account the good stability of the complexes formed with the non-nucleotide linker, corresponding fluorescent labeled probes were utilized to monitor hybrid formation by measuring fluorescence anisotropy, r.

Fluorescein-labeled (F-Probe) oligonucleotides were synthesized according to the scheme shown in figure 1 and procedure given in experimental part.

Ethylenediamine was used as a tether. It was introduced via a phosphoramidate linkage, which was formed by oxidation of a hydrogen phosphonate intermediate in CCl_4 solution. The modified oligonucleotides were subjected to labeling by a primary amine

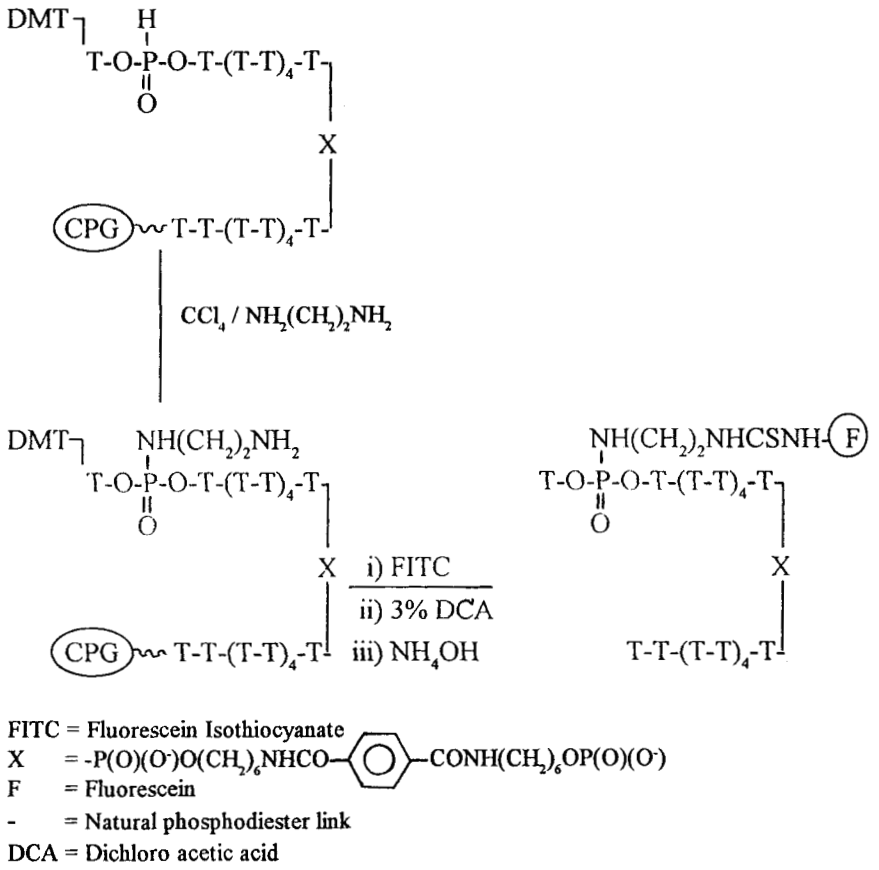

FIG. 1

orienting reagent fluorescein isothiocyanate (FITC) on the CPG. The resulting modified oligonucleotide was cleaved from CPG and subsequently purified by RP-HPLC. Figure 2 shows RP-HPLC chromatograms of purified fluorescein-labeled oligonucleotides.

The UV-Visible absorption spectrum for the fluorescein oligonucleotide conjugate $dT_{II}X\ T_{II}$ is shown in figure 3. Absorption and emission maxima of F-Probes synthesized are summarized in Table 1.

Absorption and emission maxima of $dT_{II}\ X\ T_{II}EF$ (EF = fluorescein ethylamine) in the presence of dA_{II} were essentially similar to those of $dT_{II}EF$ in the presence of dA_{II}. The results indicate that there are no substantial differences in the stability of

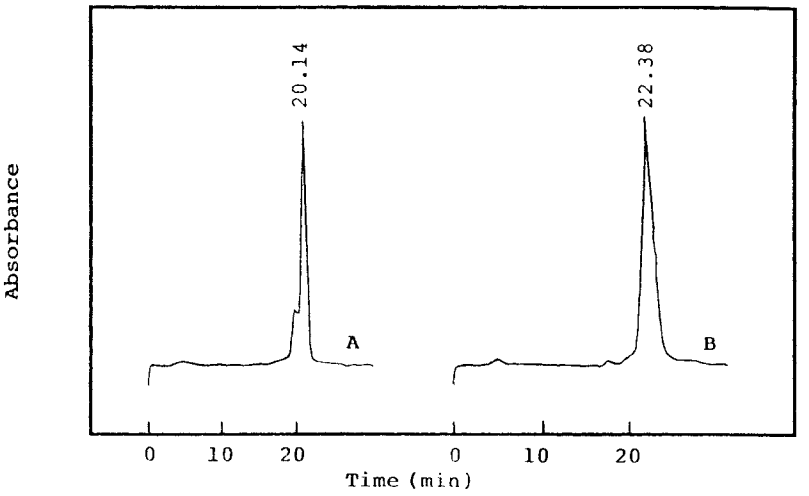

FIG. 2. HPLC chromatogram of fluorescein-labeled A- - > dT_{II} EF and B- - > dT_{II} X T_{II} EF by using column Nucleosil RP-C_{18} (10 μm) 100 mm x 2.1 mm from Altech using a CH_3CN gradient (0 to 25% in 25 min) in 0.1 M Et_3NHOAC buffer (pH 7.0), UV monitor at 496 nm.

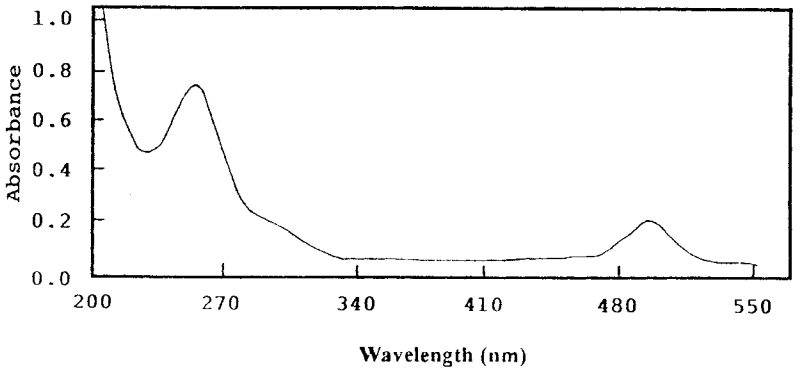

FIG.3. UV-Visible spectrum of dT_{II} X T_{II}EF in 0.1 M phosphate buffer (pH 7.0).

the F-Probe hybrids. In order to study whether fluorescein derivatives have a tendency to intercalate spontaneously between base-pairs of hybrids, EF was mixed with dT_{II}-dA_{II} hybrid, which showed no appreciable change in either r or in relative quantum yields, indicating that EF behaves independently of the hybrid present (Table). The melting temperature Tm, of dT_{II}EF-dA_{II} (22°C)

TABLE 1 - Spectroscopic characterization of F-Probes

Expt. No.	F-Probe	Absorption Spectra	Fluorescence Spectra			
		λ_{max}(nm) (visible)	λ_{max}(nm) (excitation)	λ_{max}(nm) (emission)	Relative quantum yield	Fluorescence anisotropy (r)
1.	dT2EF	490.4	493.0	515.2	1.00	0.019
2.	dT_{II} EF	494.3	496.0	520.0	1.07	0.042
3.	dT_{II} EF + dA_{II} (1 eq.) (1 eq.)	495.0	496.8	520.8	0.91	0.052
4.	dT_{II} EF + dA_{II} (2eq.) (1 eq.)	494.7	496.5	520.4	0.90	0.063
5.	EF + dT_{II} + dA_{II}	492.7	491.2	514.4	0.79	0.020
6.	dT_{II} X T_{II} EF	492.8	494.3	515.9	1.17	0.024
7.	dT_{II} X T_{II} EF + dA_{II}	493.7	493.7	515.3	1.08	0.209
8.	dT_{II} X T_{II} EF + dC_{II}	492.4	494.1	515.6	1.04	0.030

Absorption Spectra : [F-Probe] = [Oligonucleotide] = 2.5 μm;
0.1 M Phosphate buffer (pH = 7.0); r.t.
Fluorescence Spectra : [F-Probe] = [Oligonucleotide] = 0.1 μm;
0.1 M Phosphate buffer (pH = 7.0); 20°C
Relative quantum yields were calculated on the basis of T2EF.

is almost same as that of dT_{II}-dA_{II} while Tm of dT_{II} X T_{II}EF-dA_{II} (55°C) was slightly higher than that of dT_{II} X T_{II}-dA_{II} (54°C). These results indicate that no substantial differences exist in EF ability to intercalate into triplexes. This is perhaps due to the fact that fluorescein is not known to be a good intercalator.

Fluorescence anisotropy, r, reflects the molecular motion of fluorescent materials in solution[6]. Anisotropy increases when the molecular motion of fluorescent materials in solution becomes slow. r values of EF-Probes containing non-nucleotide linker were measured in the absence and presence of their complementary oligonucleotides and are summarized in Table. The dT_{II}EF has anisotropy of 0.042 (Expt. No. 2) while $dT_{II}XT_{II}$EF has anisotropy which is comparatively low (0.024, Expt. No. 6), presumably due to increase in the overall degree of freedom

due to hexamethylene units in the linker. However, an appreciable increase in anisotropy value (r = 0.209, Expt. No. 7) was observed upon the use of the non-nucleotide linker in the F-Probe over the use of the simple F-Probe without non-nucleotide linker (r = 0.052, Expt. No. 3, r = 0.063, Expt. No. 4). Whereas only slight change in r value was observed with dC_{II}, which is not with complementary to $dT_{II}EF$ (r = 0.030, Expt. No. 8). The appreciable enhancement in r in triplex containing non-nucleotide linker is due to its increased stability compared to triplex devoid of linker.

The facts that fluorescein hardly intercalates between base-pairs and that the relative quantum yields do not change in conditions studied here, eliminate the possibility that the anisotropy changes may be due to other factors aside from the hybrid formation (Expt. No. 5). These observations clearly indicate that anisotropy measurements of fluorescent oligonucleotide containing non-nucleotide linkers as described here readily leads to the monitoring of the presence of a target nucleic acid sequence in solution. Conclusively, the results obtained in this report indicate that the fluorescence anisotropy, r, can be an excellent index to monitor triplex formation in solution.

EXPERIMENTAL

Fluorescein isothiocyanate (FTIC, isomer I) and ethylene diamine were purchased from Aldrich Chemical Co., U.S.A. and 2'-deoxynucleoside loaded CPG, nucleoside H-phosphonates and cyanoethyl phosphoramidites were obtained from Glen Research Corpn., U.S.A. Fluorescein ethylamine (EF) was synthesized as reported earlier[12].

Fluorescence measurements were done on Perkin-Elmer model LS50B spectrometer equipped with a polarization accessory (Part No. L225 0100). The fluorescence was measured at 520 nm using an excitation wavelength of 480 nm.

Thermal melt (Tm) analyses were performed on a Perkin Elmer Lambda 2 spectrophotometer. The change in absorbance was measured at 260 nm with a temperature ramping of 0.5°C/min. Tm specifies the temperature at midpoint of the region of maximum slope in the plot of normalized absorbance at 260 nm Vs temperature. The total oligonucleotide strand concentration was 2.0 μm in 10mM phosphate buffer, pH 7.0 (No NaCl).

Synthesis of oligodeoxynucleotides : Oligodeoxynucleotides were synthesized on the 1.0 μ mol scale with an automatic DNA

Synthesizer (Pharmacia GA Plus) using phosphoramidite protocol provided by the manufacturer.

Synthesis of non-nucleotide linker : The non-nucleotide linker [$DMTO(CH_2)_6NHCOC_6H_4CONH(CH_2)_6OP(O)(O^-)$] was prepared from 6-aminohexanol and terephthaloyl chloride as reported earlier[11].

Synthesis of fluorescein-labeled oligonucleotide sequences : Oligodeoxynucleotides were synthesized on CPG support using nucleoside cyanoethyl phosphoramidite reagents for addition of nucleoside units, hydrogen phosphonate reagent for addition of linker and nucleoside H-phosphonate for internucleotide H-phosphonate linkage. Conventional protocols for syntheses with phosphoramidite reagents[13] and hydrogen phosphonate reagents[14] were followed, starting with 1.0 μ mol of loaded nucleoside. Couplings were carried out manually by syringe technique[15]. In the internucleotide H-phosphonate linkage, the introduction of fluorescein was carried out by oxidative coupling with ethylene diamine in CCl_4 (1.5 M, 0.4 ml, 20 min) as tether followed by subsequent labeling of the primary amine moiety by fluorescein isothiocyanate [0.5 ml, 10 mM in 100 mM carbonate buffer (pH = 9)-DMF (20%, v/v) in dark at room temperature for 48 h].

The oligonucleotides, worked up in a conventional manner and isolated by reverse phase HPLC, were better than 95% pure as confirmed by HPLC analysis.

ACKNOWLEDGEMENT

The financial assistance from the Department of Biotechnology, New Delhi, is gratefully acknowledged. We are thankful to Dr. K.N. Ganesh, Dr. A.A. Natu and co-workers, NCL, Pune for encouraging discussions and for assistance with the fluorescence experiments.

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Received November 19, 1995
Accepted July 12, 1996