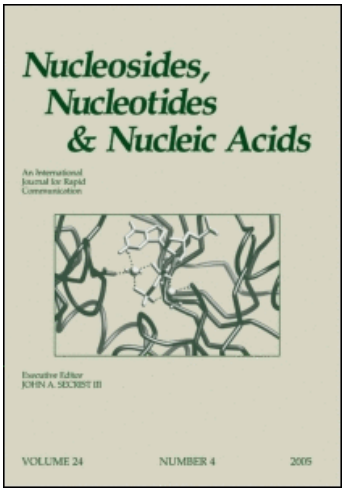

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SPIN-LABELED OLIGONUCLEOTIDES SITE SPECIFICALLY LABELED AT THE INTERNUCLEOTIDE LINKAGE. SEPARATION OF STEREOISOMERIC PROBES AND EPR SPECTROSCOPICAL DETECTION OF HYBRID FORMATION IN SOLUTION

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ABSTRACT: Described herein is the synthesis, separation of stereoisomers, hybridization, and EPR behavior of spin-labeled oligonucleotides site specifically labeled at the internucleotide linkage with 4-amino-TEMPO. The results obtained indicate that the spin-labeling method will provide a DNA probe useful for detecting hybrid formation with the complementary segment in solution.

To detect specific nucleic acids sequences or genetic defects related to serious diseases, the DNA probe method is predicted to become one of the most important research targets in future medicine[1, 2]. In this technique, a labeled oligonucleotide is used as a probe. In spite of the great need for this method, there have been two major problems preventing the actualization. First, radioactive labels have been used exclusively so far. Radioactive labels cause in clinics many problems concerning human safety. Secondly, in the currently adopted protocol, to detect hybrid formation between probes and the target, probes remaining unbound should be separated from the bound (referred to B/F separation). The B/F separation consists of skill-requiring and complicated multi steps, making this method impractical in ordinary clinics. In order to overcome these problems, there have been many efforts from a number of research groups. For the first problem, many procedures to modify oligonucleotides with non-radioactive labels such as enzymes[3], fluorescent reagents[4,5] and electron spins[6-14] have been explored. For the second, several techniques to detect hybrid formation in

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Dedicated to the late Professor Tohru Ueda.

solution have been demonstrated[15-20]. By combining above two techniques, a DNA probe method without the above problems will be obtained.

One of the most promising labelings is spin labeling. This trial started at 1988 when independently, two groups demonstrated successful detection of hybridization by the use of two different types of chemically synthesized spin-labeled oligonucleotides. One was based on labeling C5 of the pyrimidine base moiety (thymine)[10] and the other introduced labels onto an internucleotide phosphate linkage[12].

For the former technique, several research groups have explored the improvement of chemical syntheses of labeled oligonucleotides as well as label-carrying monomers. So far, however, only C5 of pyrimidine bases has been a labeling site almost exclusively. Also, the preparation of the monomers is based on the multi step syntheses with relatively low yields. These disadvantages may hamper wide use, although EPR spectra of these spin-labeled oligonucleotides changed characteristically on hybrid formation.

The latter method seems to overcome the above problems because hydrogen phosphonate (H-phosphonate) monomers necessary for the labeling are already commercially available and can be coupled with automated synthesis and because therefore, labels can be attached to any internucleotide linkage: it should be noted that oxidization of the H-phosphonate linkage with amino-modified electron spins results in successful introduction of spin-labels[21-23]. Our previous studies have demonstrated that oligonucleotide probes labeled with electron spins[12,17] as well as with fluorescent[19] moieties were prepared *via* H-phosphonate linkage with reasonably high yields without any undesirable inactivation of the labels and that hybrid formations between the probes and the target could be monitored by observing the line broadening and the change in the fluorescence anisotropy, respectively.

In this study, our aim is placed on exploring the EPR behavior of spin-labeled oligonucleotides site specifically labeled at the internucleotide linkage upon hybrid formation, in particular on difference in hybrid formation between the stereoisomers which are formed on introduction of spin labels to the H-phosphonate linkage. We separated the stereoisomers and examined their hybridization manners using UV melting curves, circular dichroism (CD) spectrophotometry and electron paramagnetic resonance spectroscopy (EPR). The results shown here will be useful for the further improvement of the DNA probe method coupled with electron spins.

MATERIALS AND METHODS

Fully protected deoxyribonucleoside phosphoramidites and reagents for the oligonucleotide synthesis were obtained from Milligen Biosearch (Burlington, MA, USA) and deoxyribonucleoside H-phosphonates (triethylammonium salt) were from

Sigma Chemical Company (St. Louis, MO, USA). 4-Amino-2,2,6,6-tetramethylpiperidine-N-oxyl (4-amino-TEMPO) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA) and stored in CCl_4 dried over CaH_2. Pivaloyl chloride was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CH_3CN and CCl_4 were dried over molecular sieves 4A. Other reagents were used without further purification. Oligonucleotides used as models of the target DNA were synthesized by the solid-phase phosphoramidite protocol on a Milligen Biosearch Cyclone Plus DNA Synthesizer and purified by RPLC.

Spin-labeled oligonucleotides.

Spin-labeled oligonucleotides were synthesized by a combination of automated solid-phase phosphoramidite protocol and a syringe synthesis. An oligonucleotide was synthesized on controlled pore glass (CPG) using a solid-phase phosphoramidite protocol on the DNA synthesizer. The oligonucleotide bound CPG was transferred to a gas-tight syringe and washed with CH_3CN, followed by drying *in vacuo*. All modifications were carried out in the syringe. Fully protected nucleoside H-phosphonate (0.15 mL, 0.2 M) in pyridine/CH_3CN (1/1, v/v) was added to the CPG and this system was shaken vigorously for 10 sec, followed by the addition of pivaloyl chloride (0.15 mL, 1.0 M in pyridine/CH_3CN (1/1, v/v)). This system was shaken for 5 min and this condensation step was repeated to enhance the condensation yield. The CPG was washed sequentially with CH_3CN/pyridine (1/1, v/v) and CH_3CN, and dried *in vacuo*. Subsequently, a CCl_4 solution of 4-amino-TEMPO (0.2 M, 0.4 mL) was added to the CPG and this system was allowed to react for 24 hr at 55 °C. This oxidation step was duplicated. Then, the CPG was washed sequentially with CCl_4 and CH_3CN, and dried *in vacuo*. The resulting products were removed from the CPG by treatment of concentrated NH_4OH (28 %) for 1 hr at room temperature. The supernatant combined with the washings of the CPG was heated at 55 °C for 6 hr to remove the protecting groups of the bases and the solvent was removed by evaporation to dryness. The resulting crude spin-labeled oligonucleotide was dissolved in 5 % CH_3CN in 100 mM triethylammonium acetate (TEAA, pH 6.8) and purified by reversed-phase high-performance liquid chromatography (RPLC). A Tosoh CCPM pump (Tokyo) equipped with a Tosoh UV-8000 UV flow monitor (Tokyo) was utilized. Detailed chromatographic conditions appear in the figure legend. Spin-labeled oligonucleotides prepared in the present study are summarized in TABLE 1 together with their abbreviations and their complementary ordinary oligonucleotides used as their target. Concentrations of oligonucleotides were determined based on UV measurements in

TABLE 1. Sequences of spin-labeled oligonucleotides and oligonucleotides and introduction yield of 4-amino-TEMPO.

	abbreviation	sequence*	introduction yield (%)
spin-labeled oligonucleotide	SP1	dTp*T	65
	SP2	dTp*TTTT	71
	SP3	dTp*TTTTTTTTT	67
	SP4	dGp*GGAATTCGT	72
	SP5	dCp*CGCCAGGCA	72
	SP6	dTp*CGGCATGGG	75
oligonucleotide	P4	dGGGAATTCGT	
	4C10	dACGAATTCCC	
	4C20	dTGATTACGAATTCCC GGGGA	
	4C30	dGACCATGATTACGAA TTCCCGGGGATCCGT	
	5C10	dTGCCTGGCGG	
	6C10	dCCCATGCCGA	

*The site where a spin-label is introduced.

which we took the molar extinction coefficients derived according to the procedures reported previously[24].

Spectroscopic measurements.

UV-monitored thermal denaturation experiments were carried out on a Shimadzu UV-260 spectrophotometer (Kyoto, Japan) equipped with a KPC-5 temperature programmer and an SPR-5 temperature controller. The monitoring wavelength was 260 nm. The rate of the temperature raise was 0.3 °C/min. Samples were dissolved in 0.1 M phosphate buffer (pH 7.0). The final concentration of the oligonucleotide was 5 μM. These samples were also subjected to other measurements, unless otherwise specified.

CD spectra were recorded on a J-720 spectropolarimeter (JASCO, Tokyo). The cell temperature was controlled by an RTE-100 thermostated water bath (Neslab, Newington, NH, USA). The path length of the cell was 10 mm.

EPR measurements were performed on a JEOL PE-3X ESR Spectrometer (Tokyo) with an X-band cavity resonator at 22 °C (controlled room temperature). An aqueous quartz flat cell (outer dimensions being 45X10X2 mm, LABOTEC, Tokyo) was used. Prior to the measurements for hybrid formation, the solution containing both a spin-labeled oligonucleotide (final concentration: 10 μM) and a target oligonucleotide (final concentration: 10 μM) was initially maintained at 85 °C in a water bath and then gradually cooled down to the room temperature. To remove molecular oxygen, samples

were degassed by bubbling argon gas for 1 min. EPR spectra were recorded at 9.56 GHz resonant frequencies and with 100 kHz field modulation of 0.063 mT amplitude and nonsaturating microwave power of 10 mW.

RESULTS AND DISCUSSION

Preparation of spin-labeled oligonucleotide.

Spin-labeled oligonucleotides were synthesized according to the procedure shown in FIG.1. 4-Amino-TEMPO was subjected to oxidation of an H-phosphonate intermediate and bound to the oligonucleotides *via* the phosphoramidate linkage. The introduction yields of 4-amino-TEMPO for several oligonucleotides are listed in TABLE 1. Compared to the yields for other primary and secondary alkyl amines, these values were slightly lower. This may be due to steric hindrance by the ring structure of 4-amino-TEMPO. The aminoxyl radical was not inactivated throughout this oxidization as well as in concentrated NH_4OH used for the deprotection.

Since oxidation of an H-phosphonate linkage with amines leads to a set of phosphoramidate stereoisomers, RPLC was applied to isolate each stereoisomer of the spin-labeled oligonucleotides. Shown in FIG.2a is a typical RPLC chromatogram obtained from **SP4** before detritylation, where two distinct peaks appear at the elution time of 35.2 and 36.0 min: it should be noted that these isomers eluted more closely after detritylation, as shown by the chromatogram of the detritylated oligomers (see FIGS.2b and c). All of the spin-labeled oligonucleotides were, therefore, separated with the dimethoxytrityl (DMTr) group on for the following experiments. In this study, the isolated stereoisomers are refered to isomer 1 and 2, according to the order of the elution. DMTr groups were subsequently removed in 80 % AcOH for 30 min at room temperature. During the course of the detritylation, the aminoxyl radical moiety was not inactivated. Deprotected stereoisomers were further purified by RPLC.

Spectroscopic behavior of the spin-labeled oligonucleotides in solution.

Introduction of 4-amino-TEMPO may change the solution structure of the oligonucleotides. Therefore, CD spectra were measured for all the isomeric oligonucleotides. Shown in FIGS.3a, b, and c are the CD spectra obtained from the single stranded stereoisomers of **SP4**, **SP5**, and **SP6**, respectively. The molar ellipticity measured as the maximum peak height was found to be dependent on the oligonucleotide structure, although the wave length at which maximum positive Cotton effect appeared was slightly shifted according to the samples. For example, for **SP4**, the ellipticity around 270 nm was almost twice larger for the isomer 2 than that for the isomer 1. The analogous tendency was also observed for the stereoisomers of **SP5**.

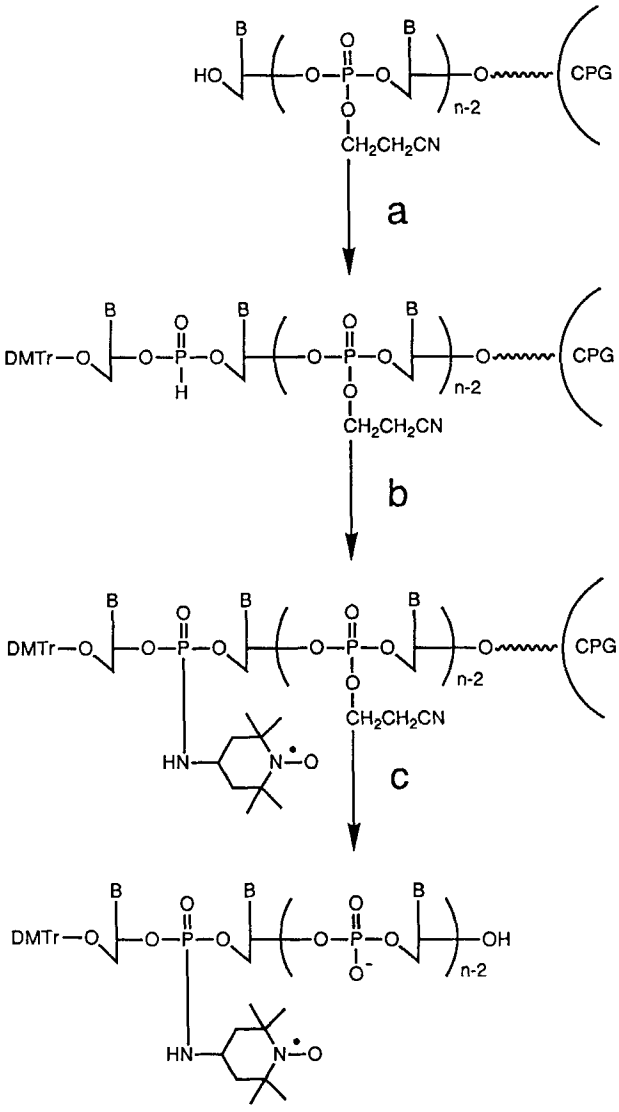

FIG.1 Synthetic scheme of spin-labeled oligonucleotides.
Reagents used are (a) fully protected deoxyribonucleoside H-phosphonates (triethylammonium salt) and pivaloyl chloride, (b) 4-amino-TEMPO / CCl_4, and (c) concentrated NH_4OH.

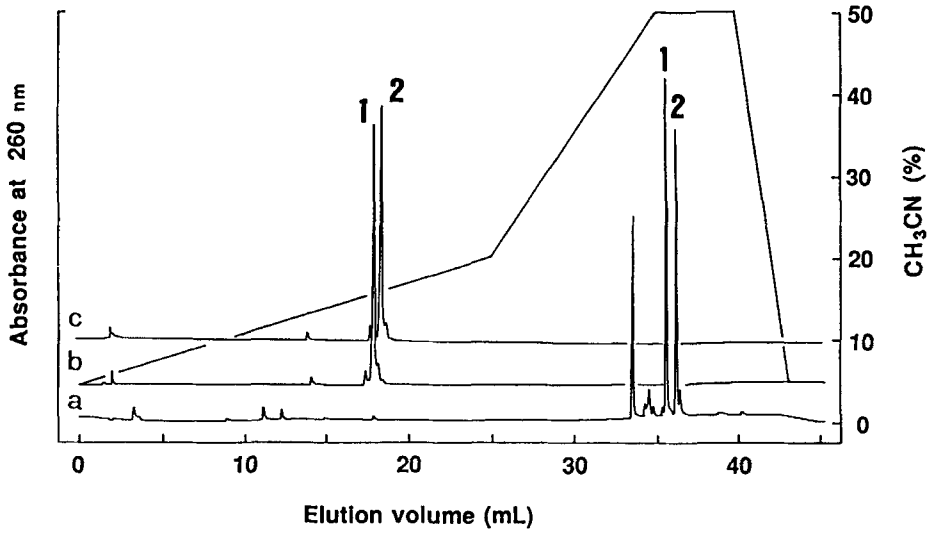

FIG.2 RPLC chromatogram obtained from (a) a crude mixture of the stereoisomers of SP4 with DMTr on, (b) the detritylated SP4 from fraction 1, and (c) the detritylated SP4 from fraction 2.
Chromatographic conditions: column, Wako-Pak C18 (5 μM, 4X150 mm); eluent, (low) 5 % CH_3CN in 0.1 M TEAA and (high) 50 % CH_3CN in 0.1M TEAA; gradient, as indicated in the figure; detection: 260 nm.

For both the stereoisomers of **SP6**, however, almost the same CD spectra were obtained. These results suggest that the solution structures of some spin-labeled oligonucleotides are different between their stereoisomers. Such structural differences were not observed by absorption spectrophotometry.

To see if the structural difference corresponds to the EPR line shape of spectra, the following measurements were performed using the same samples. Represented in FIGS.4a and b are EPR spectra obtained from isomer 1 and 2 of single stranded **SP4**, respectively. Both the spectra consist of a triplet due to the aminoxyl nitrogen (a_N=1.71 mT). To characterize the line shape obtained from each sample, the ratios of I_{+1}/I_0 and I_{-1}/I_0 were used. The values of I_{+1}, I_0, and I_{-1} correspond to the intensity of the +1, 0 and -1 components of the triplet. As summarized in TABLE 2, I_{-1}/I_0 was obviously different between the stereoisomers of **SP4** and **SP5** while I_{+1}/I_0 was not. No difference was observed for the stereoisomers of **SP6**. As discussed earlier in this text (see FIG.3), CD spectra obtained from **SP4** and **SP5** showed characteristic difference between the stereoisomers. The isomer 2 of **SP4** and **SP5** which showed larger CD

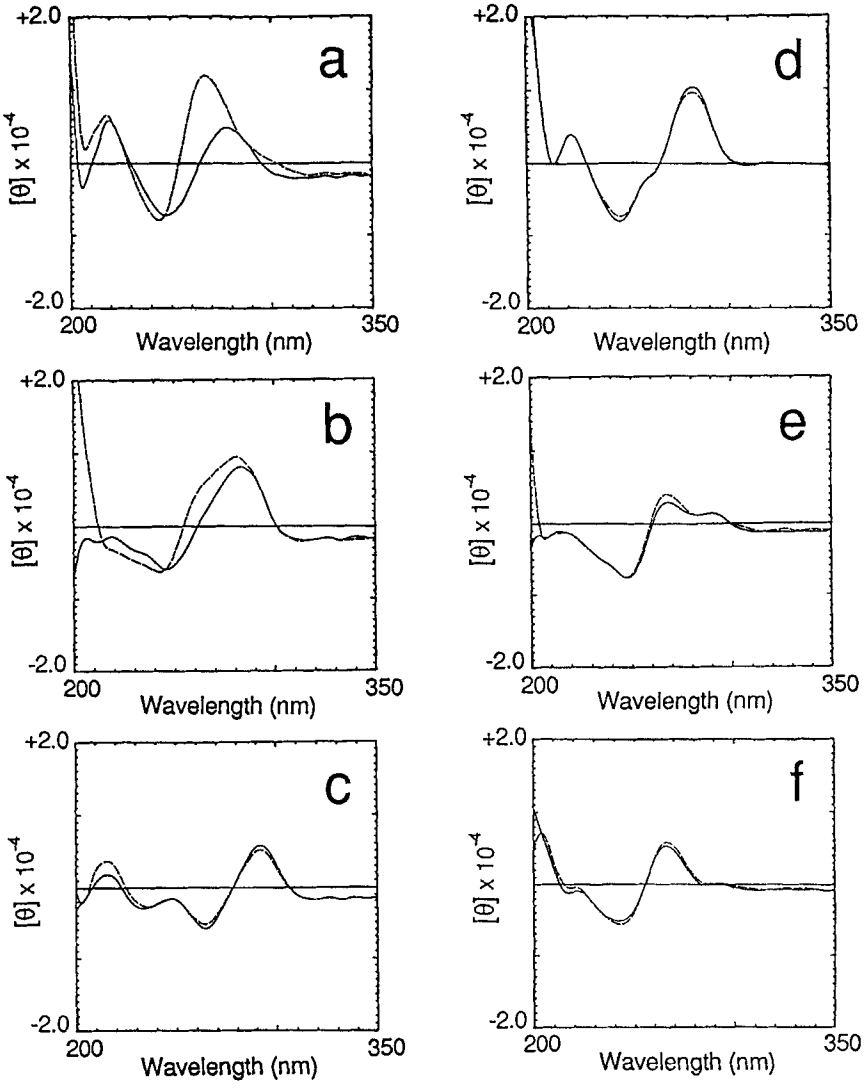

FIG.3 CD spectra obtained from single stranded stereoisomers of (a) **SP4** , (b) **SP5**, and (c) **SP6** and from the hybrids of the isomers (d) **SP4** + **4C10**, (e) **SP5** + **5C10**, and (f) **SP6** + **6C10**. Solid and broken lines correspond to isomer 1 and 2, respectively.
Conditions: concentration of the oligonucleotides: 5 μM; buffer, 0.1 M phosphate (pH 7.0); temperature, 20 °C.

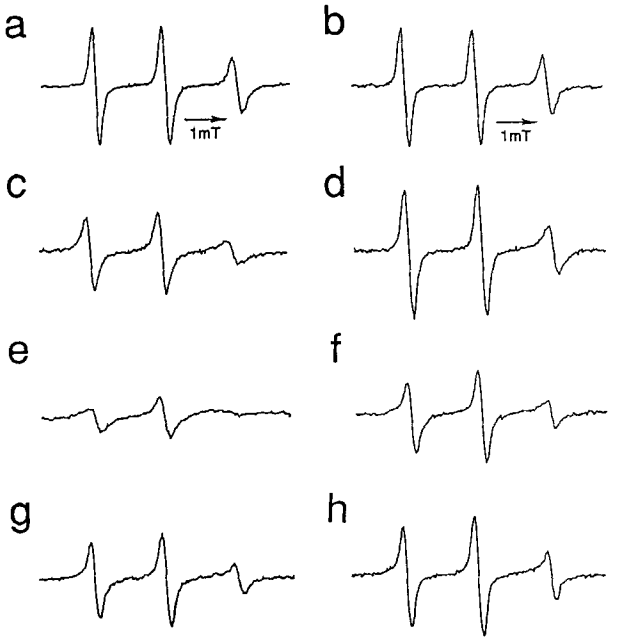

FIG.4 EPR spectra obtained for **SP4**. Samples were (a) isomer 1, (b) isomer 2, (c) isomer 1 + **4C10**, (d) isomer 2 + **4C10**, (e) isomer 1 + **4C20**, (f) isomer 2 + **4C20**, (g) isomer 1 + **4C30**, and (h) isomer 2 + **4C30**.
Conditions: concentration of the oligonucleotides: 10 μM; buffer, 0.1 M phosphate (pH 7.0): temperature: 22 °C
EPR settings: modulation, 0.063 mT; gain, X2000; response, 3 sec; sweep time, 64 min.

intensity showed smaller line broadening in the EPR spectra. These results may indicate that the more compact structure (corresponding to apparent molecular size in solution) caused by base stacking is responsible for the smaller broadening. To see that this is the case, the ratio of I_{+1}/I_0 and I_{-1}/I_0 indicative of the molecular motion was plotted against the molecular weights of the oligonucleotides. As shown in FIG.5, the value of I_{-1}/I_0 decreased with increment in the molecular weight of the single stranded spin-labeled oligonucleotides while the other was not influenced. These results suggest that the I_{-1}/I_0 ratio reflects the apparent molecular size in solution of the single stranded spin-labeled oligonucleotides, which is related to the extent of the global tumbling of spin-labeled oligonucleotides.

TABLE 2. EPR characteristics of the spin-labeled oligonucleotides.

spin-labeled oligonucleotide		molecular weight	I_{+1}/I_0	L_1/I_0
4-amino-TEMPO		171	1.01	0.93
SP1	isomer 1	700	1.00	0.75
	isomer 2		0.99	0.76
SP2	isomer 1	1609	1.02	0.63
	isomer 2		1.04	0.61
SP3	isomer 1	3125	1.01	0.56
	isomer 2		1.03	0.55
SP4	isomer 1	3228	1.02	0.44
	isomer 2		1.03	0.51
SP4+4C10	isomer 1	6191	0.89	0.28
	isomer 2		0.96	0.38
SP4+4C20	isomer 1	9366	0.59	0.19
	isomer 2		0.75	0.29
SP4+4C30	isomer 1	12406	0.82	0.32
	isomer 2		0.85	0.39
SP5	isomer 1	3143	1.00	0.37
	isomer 2		1.05	0.49
SP5+5C10	isomer 1	6169	0.93	0.28
	isomer 2		0.99	0.42
SP6	isomer 1	3229	1.00	0.42
	isomer 2		1.02	0.43
SP6+6C10	isomer 1	6184	0.99	0.36
	isomer 2		1.03	0.42

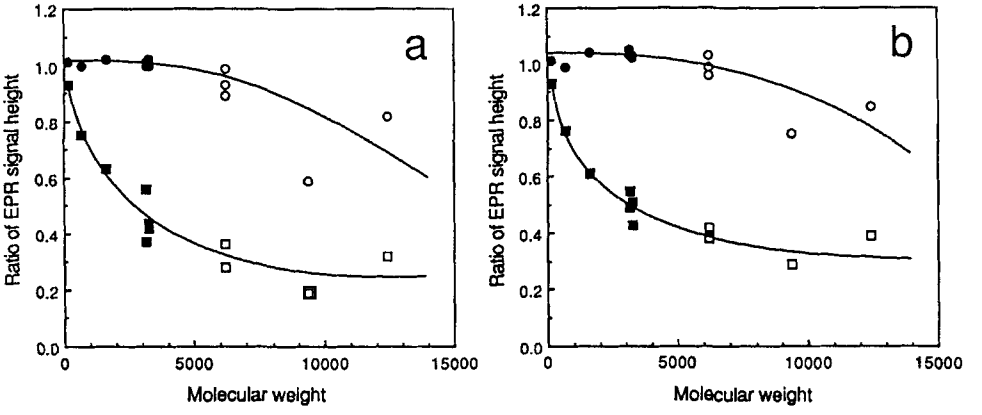

FIG.5 Plots between the molecular weight of the spin-labeled oligonucleotides and the ratios of I_{+1}/I_0 and L_1/I_0 for (a) isomer 1 and (b) isomer 2.
I_{+1}/I_0, (●) single and (○) double strands; L_1/I_0, (■) single and (□) double strands.

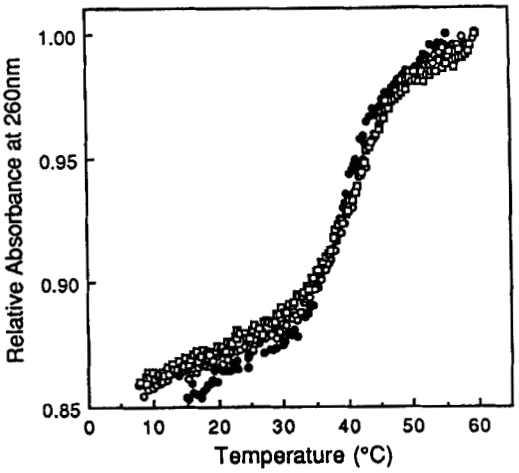

FIG.6 UV melting curves obtained for (●) P4 + C10, (o) SP4 (isomer 1)+ 4C10, and (□) SP4 (isomer 2) + 4C10.
Conditions: concentration of the oligonucleotides: 5 μM; buffer, 0.1 M phosphate (pH 7.0); monitoring wavelength, 260 nm.

Hybrid formation of spin-labeled oligonucleotide.

In order to study the hybrid formation between the spin-labeled oligonucleotides and their complementary oligonucleotides, UV melting curve measurements were carried out. Depicted in FIG.6 are melting curves obtained for the stereoisomers of **SP4**. As a reference, a curve obtained for the hybrid between ordinary oligonucleotides, **P4** and **4C10**, accompanies in the same figure. As is seen in the figure, the melting curves obtained for the stereoisomers of **SP4** using **4C10** as a target are almost identical with that obtained for the reference sample, and between the stereoisomers no difference was observed for both the curves: the duplex melting temperatures obtained from the melting curves are 41.0 °C for both isomer 1 and 2 in the presence of their complementary strand (**4C10**) and 40.0 °C for the reference. The extent of hyperchromicities was almost equal between these samples. Experiments for **SP5** and **SP6** showed a similar tendency. These results indicate that the spin-labeled oligonucleotides hybridize with their complementary oligonucleotides and that the 4-amino-TEMPO bound on the internucleotide linkage does not interfere with the hybrid formation.

The CD spectra obtained from the stereoisomers of **SP4, SP5,** and **SP6** in the presence of their complementary strands of **4C10, 5C10,** and **6C10** are represented in FIGS.3d, e, and f, respectively. These spectra are characteristic of those of hybrids in the

B conformation[25]. Different from the single stranded stereoisomers, these hybrids did not show differences in ellipticity between the stereoisomers, suggesting that CD spectra of the hybrids do not reflect the structural differences observed for the single stranded stereoisomers.

EPR may show differences between the hybrids of the stereoisomers since this technique is extremely sensitive to the mobility of the samples which corresponds to their apparent size in solution. EPR spectra obtained for isomer 1 and 2 of **SP4** in the presence of their complementary strands with different chain length (4C10, 4C20, and 4C30) are lined up in FIGS.4c, e, and g and d, f, and h, respectively. Compared to the spectra of the single-stranded molecules (FIG.4a and b), the EPR line was broadened in the presence of the complementary strands. This broadening increased with increase in the chain length of the target oligonucleotides and this trend was different between the stereoisomers. Similar results were obtained for **SP5**. However, no change was observed for the stereoisomers of **SP6** although hybrid formation with the complementary sequence was detected for them by the melting curves. The reason for this unusual EPR behavior of SP6 remains unsolved but this result will be cautionary for designing spin-labeled probes or presumably for selecting the target sequences. The above results imply that using suitably designed probes, EPR can monitor the slight difference in the hybrid and therefore will be a powerful tool to distinguish the 1:1 hybrid between the probe and the target DNA. To evaluate the line broadening, the ratios I_{+1}/I_0 and I_{-1}/I_0 were calculated from the EPR spectra and are summarized in TABLE 2. Since the molecular weight may partly contribute to the line broadening, the molecular weights of the hybrids were plotted against the values for the samples. The profile is shown in FIG.5 where the expected relationship is obvious: I_{+1}/I_0 was sensitive to the hybrid formation while I_{-1}/I_0 was to the molecular weights of the single stranded oligonucleotides.

Conclusively, when the probes are designed suitably and the targets are selected properly, spin-labeling of oligodeoxyribonucleotides at the internucleotide linkage will provide DNA probes useful for detecting hybrid formation in solution. Numerical values calculated from the EPR spectra, I_{+1}/I_0 and I_{-1}/I_0, will be a good monitor for the increase in the molecular weight on hybrid formation. Separation of the stereoisomers of the spin-labeled oligonucleotides will give rise to more accurate detection.

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