Interaction of Antisense Oligonucleotides with DNA and Proteins Studied by Fluorescence Polarization Spectroscopy

Akira Murakami, Misuzu Nakaura, Hiroki Uematsu
Naoko Fujimura, Reiko Iwase, Tetsuji Yamaoka
Department of Polymer Science and Engineering
Kyoto Institute of Technology
Matsugasaki, Sakyo-ku, Kyoto, Japan

1. Introduction

During past decade genetic information related to diseases such as AIDS and various types of cancer have been accumulated. For example, the complete nucleotide sequences of c-myc oncogene and HTLV-III has been elucidated, respectively (1,2). Aiming the gene therapy of such diseases based on genetic information, regulation of gene expressions essential for those diseases have been attempted (3-10) using oligodeoxyribonucleotides (ODN) and their analogs. This concept, so-called antisense strategy, is the first case to successfully challenge in curing diseases with theoretically designed and synthesized chemical materials. Among reports, however, there exist some contradictory results. Some cases showed sequence specific regulations (11,12) and some cases were found to be sequence non-specific (13). Furthermore, our knowledge of the basic mechanisms of the antisense method involved in internalization of antisense molecules by cells (14-16), intracellular traffic (17-19), and interaction with RNAs (20), DNAs or proteins, is still fragmentary. Many reports have concerned mechanisms of antisense gene regulation and it has been regarded that interaction and following complex formation of antisense molecules with substances in a body might play important roles on the antisense effects. Antisense molecules may interact with serum proteins, blood cells, tissues, receptors, polysaccharides, endoplasmic reticulums, ribosomes, nuclear matrices, and so on, as well as various types of nucleic acids.

Fluorescent-labeled antisense molecules have been utilized to study the uptake mechanism by cells, and the localization inside cells. As the next stage for the antisense research, it is quite important to clarify the mechanisms in molecular resolution. In this paper, we describe studies on the interactions

of four types of antisense molecules (OPT, ODN, oligonucleoside methylphosphonate (OMP) and peptide nucleic acid (PNA)) with oligonucleotides and serum proteins such as human serum albumin (HSA), human immunoglobulin (HIG), and human transferrin (HTF). Those proteins, as well as blood cells and tissues, are presumably the first substance for antisense molecules to interact when administered into a body. The interaction was studied with the liquid-phase DNA-probe method. The method includes fluorescence depolarization spectroscopy using fluorescein-labeled antisense molecules (F-AS) (21). The spectroscopic method was proven to be potent to monitor the molecular motion of biological substances in solution (22) (Mejillano et al., 1989) without disturbing their structures and biological functions, and to study interactions of antisense molecules with substances in a body.

2. Experimental procedures

2.1. Materials

Reagents for the oligonucleotide synthesis were obtained from Applied Biosystems Inc. (Foster City, CA, USA). Fluorescein isothiocyanate (FITC, type 1), human serum albumin, human immunoglobulin, and human transferrin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Other reagents were purchased from Wako Pure Chemicals, Inc. (Osaka, Japan) and used without further purification.

2.2. Assay conditions

Absorption spectra were measured in 0.1 M phosphate buffer (pH 7.0) at 20°C by a Shimadzu UV-260 spectrophotometer (Kyoto, Japan). A high performance liquid chromatography system (LC-6A, Shimadzu Co. Kyoto, Japan) equipped with a reversed phase column (C18, 4.6∅ x 150 mm, Ultron N-C18, Shinwa Chemical Industries Inc., Kyoto, Japan) was used to analyze and purify labeled-antisense molecules. The elution buffer was acetonitrile (AN) in 0.1 M triethylammonium acetate (TEAA, pH 7.0) and the gradient was linear as follows; AN5% (0 min) to AN20% (20 min), then 50% (30 min). Fluorescence spectra were measured in 0.1 M phosphate buffer (pH 7.0) at 20°C with a fluorescence spectrophotometer (RF-5000, Shimadzu Co.) equipped with a set of polarizing plates and with a thermal controller. The excitation wavelength used was 480 nm with 5 nm bandwidth and the emission was monitored at 520 nm with 5 nm bandwidth.

Fluorescence life times of F-AS were determined by a single photon counting method on NAES-550 (Horiba, Kyoto, Japan) equipped with a nano second flash lamp filled with hydrogen and with a thermal controller in 0.1 M phosphate buffer (pH 7.0) at 20°C. Excitation light was passed through a band-path filter (center frequency: 340 nm). The fluorescence decay curves

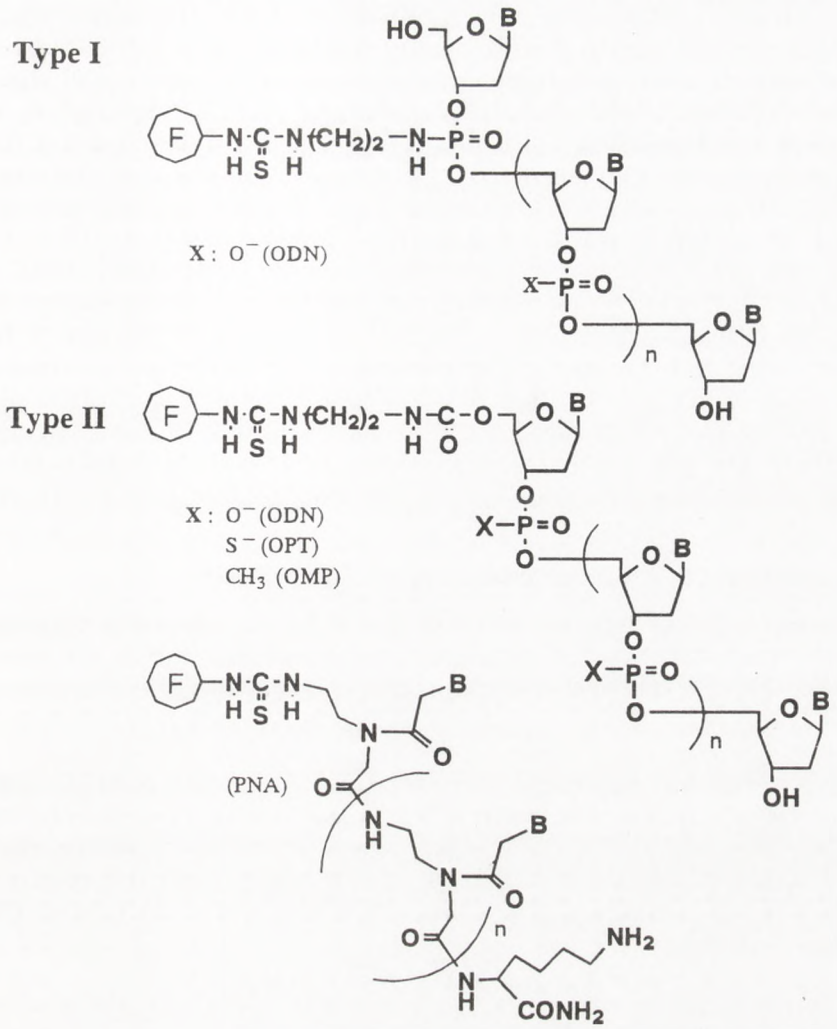

Fig. 1. Structures of fluorescent-labeled antisense molecules.

were analyzed by using a deconvolution program equipped with the apparatus. For anisotropy studies, F-AS, oligonucleotides and serum proteins were diluted to various concentrations and the solutions were incubated at 4°C overnight prior to the anisotropy measurements.

2.3. Preparation of Fluorescein-labeled Antisense Molecules

Oligodeoxyribonucleotides were synthesized on CPG-support carrying a fully protected nucleoside (1μmol) by a phosphoramidite protocol with an ABI DNA synthesizer (Type-381). F-ODN (Type I) was prepared according to

reported procedures (21). For the syntheses of OPT, the iodine-oxidation step in the protocol was replaced by the thiolation with tetraethylthiuram disulfide (15 min) (23). N,N'-carbonyldiimidazole (CDI, 300 μmol) dissolved in 1mL of dry dioxane was introduced into the gas-tight syringe in which CPG-support carrying oligonucleotides was packed. After activation for 1 h at room temperature (24) and washing with acetonitrile and dioxane, the support was treated with ethylenediamine in dioxane (0.1 M) and allowed to react at room temperature for 2 h. The support was them treated with FITC solution (50 mM, 0.5 mL, 100 mM carbonate buffer (pH9)-DMF (20%, v/v) solution) and allowed to react in the dark at room temperature for 48 h. The support was treated with concentrated NH_4OH at 55°C for 8 h, and the supernatant was evaporated to dryness. The residue was subjected to reversed phase HPLC purification. UV-VIS (0.1 M phosphate buffer, pH 7.0, 20°C): F-ODN, λmax = 493 nm; F-OPT, λmax = 493 nm; Fluorescence (0.1 M phosphate buffer, pH 7.0, 20°C, λex = 490 nm): F-ODN, λem = 516 nm, F-OPT: λem = 517 nm, F-OMP, λem = 516 nm, F-PNA: λem = 516 nm.

2.4. Fluorescence Depolarization Spectroscopic Studies

Fluorescence anisotropy (**r**) was calculated by the following equation,

$$\mathbf{r} = [I_{VV} - I_{VH}(G)] / [I_{VV} + 2I_{VH}(G)] \quad [1]$$
$$(\ G = I_{HV} / I_{HH}\ )$$

where I_{VV} and I_{VH} represent the emission intensities parallel and perpendicular to the polarization direction of excitation, respectively, and G value is a correction factor for the transmission efficiency of the emission monochromater. Fluorescence anisotropy is correlated with the apparent hydrated molecular volume (v_{app}) by Perin-Weber equation as follows (25);

$$1 / \mathbf{r} = 1 / \mathbf{r}_o\ (1 + kBT\tau / v_{app}\eta) \quad [2]$$

(r_o: limiting anisotropy value, kB: Boltzman constant,
T: absolute temperature, η:viscosity of the solution)

The binding features between F-AS and proteins were evaluated by Scatchard plots. The binding equilibrium was assumed to be;

$$[\text{F-AS}] + n[\text{Protein}] = [\text{F-AS=n(Protein)}] \quad [3]$$

and dissociation constant (Kd) can be expressed as,

$$[\text{F-AS}]_{bound}/[\text{F-AS}]_{total} = n[\text{Protein}] / \{Kd + [\text{Protein}]\} \quad [4]$$

where $[\text{F-AS}]_{total}$ and $[\text{F-AS}]_{bound}$ were the total concentration of F-AS, and concentration of F-AS bound to protein, respectively. Here, in the sol-

ution containing several fluorescent species, an observed anisotropy value can be expressed as

$$\mathbf{r} = \Sigma \mathbf{r}_i \, f_i \qquad [5]$$

where $\mathbf{r}_i$ is an anisotropy value of i-th component and f_i is the molar fraction of i-th component. Therefore, using anisotropy values, [4] can be expressed as

$$[\text{F-AS}]_{bound}/[\text{F-AS}]_{total} = (\mathbf{r}_{obs} - \mathbf{r}_{min}) \; / \; (\mathbf{r}_{max} - \mathbf{r}_{min}) \qquad [6]$$

$$\mathbf{r}_{obs} = \mathbf{r}_{min} + (\mathbf{r}_{max} - \mathbf{r}_{min}) \times n[\text{Protein}] \; / \; \{Kd + [\text{Protein}]\} \qquad [7]$$

where $\mathbf{r}_{obs}$, $\mathbf{r}_{min}$ and $\mathbf{r}_{max}$ were observed, minimum, and maximum anisotropy, respectively.

3. Results and discussion

When antisense molecules are administered to a body as reagents for gene therapy, they promptly interact with many biological substances such as blood cells, serum proteins and so on. Forming certain complexes with them, antisense molecules can be delivered to various organs and tissues. When they reach the target sites, antisense molecules should interact with target genes. To understand antisense mechanisms, detailed features of such interaction are to be intensively studied. We have focused on details of interactions of antisense molecules with genes or serum proteins by spectroscopic methods. The fluorescence depolarization spectroscopy is reportedly suitable to study such interactions in physiological conditions (21,26). The spectroscopic parameters, fluorescence anisotropy, **r**, which reflect the molecular rotational motion of fluorescent materials in solution (21) was adapted an index. Anisotropy values change as the molecular rotational motion of fluorescent materials in solution changes. There are several cases when **r** value increases as follows: [1] lowering temperature, [2] increasing viscosity, [3] spatial restriction (e.g. incorporation into membranes and stacking) and [4] covalent or non-covalent immobilization to the large molecule (e.g. proteins, DNAs, or membranes). Case [1] and [2] can be ruled out in this study. Case [3] and Case [4] are focused in this manuscript. In this study, as a fluorescent material, used was fluorescein that was immobilized to oligonucleotides covalently through a tether molecule. When F-AS is mixed with DNAs (or oligonucleotides) or proteins and when it interacts with those substances, the apparent molecular size (v_{app}) of the adduct should increase. In consequence, as shown in equation [2], **r** increases. These clearly suggest that the measurement of **r** readily lead to the monitoring the interaction of fluorescent materials with substances in homogeneous solution. Thus, the binding of small molecules (such as antisense molecules) to large molecules (such as large proteins) can be evaluated by the change of the anisotropy.

For this method, fluorescent-labeled antisense molecules shown in Fig. 1 were synthesized according to the reported methods with slight modifications (27). They are classified into two types according to the sites where fluorescein was introduced. Those modified oligonucleotides were purified by HPLC.

3.1. Interaction with Genes

Fluorescence anisotropy, **r**, of F-OND (Type I) was measured in the absence and presence of oligonucleotides, which are complementary or non-complementary, and is summarized in Table 1. As previously reported, when fluorescein-labeled oligothymidylates were used, **r** increased as they interact with oligoadenylates (21).

TABLE 1
ANISOTROPY CHANGE OF FLUORESCEIN-LABELED ANTISENSE MOLECULES (TYPE I) IN THE PRESENCE OF ITS EQUIMOLAR COMPLEMENTARY STRAND

Exp. No.	Sequence		Fluorescence Anisotropy	Relative Quantum Yield
	F-AS* (Type I: ODN)	Complementary Strand		
1	dT*TTTTTTTTT		0.042	1.00
2	dT*TTTTTTTTT	dAAAAAAAAAA	0.054	0.92
3	dT*CGGCATAGGG		0.042	0.78
4	dT*CGGCATAGGG	dCCCATGCCGA	0.061	0.77
5	dG*GAATTCGT		0.031	0.62
6	dG*GAATTCGT	dACGAATTCCC	0.033	0.63
7	dT*TGAATTCGT		0.042	0.99
8	dT*TGAATTCGT	dACGAATTCAA	0.055	0.81
9	dT*TTGGGAATTCGT		0.038	1.02
10	dT*TTGGGAATTCGT	dACGAATTCAA	0.043	0.96

[F-AS (Type I; ODN)] = [Complementary strand] = 0.1 μM.,
0.1 M. Sodium phosphate (pH 7.0), 20°C, λex = 490 λnm, ex = 520 nm.
*: site where fluorescein is introduced.

TABLE 2
ANISOTROPY CHANGE OF FLUORESCEIN-LABELED ANTISENSE DNA (TYPE II) IN THE PRESENCE OF ITS EQUIMOLAR COMPLEMENTARY STRAND

Exp. No.	Sequence		Fluorescence Anisotropy	Relative Quantum Yield
	F-AS* (Type II: ODN)	Complementary Strand		
11	d*ATTTTTTTTT		0.066	0.93
12	d*ATTTTTTTTT	dAAAAAAAAAT	0.089	0.95
13	d*CTTTTTTTTT		0.061	0.97
14	d*CTTTTTTTTT	dAAAAAAAAAG	0.060	0.64
15	d*GTTTTTTTTT		0.077	0.44
16	d*GTTTTTTTTT	dAAAAAAAAAC	0.090	0.33
17	d*TTTTTTTTTT		0.058	1.00
18	d*TTTTTTTTTT	dAAAAAAAAAA	0.083	0.91

[F-AS (Type II; ODN)] = [Complementary strand] = 0.1 μM.,
0.1 M. Sodium phosphate (pH 7.0), 7,5°C, λex = 490 nm, λex = 520 nm.
*: site where fluorescein is introduced.

The results suggest that the increment in **r** reflects the hybrid formation of F-AS with its complementary oligonucleotide. In order to generalize the concept, F-ASs composed of various bases were examined. In general, **r** increased as F-AS formed hybrid with its complementary oligonucleotide. However, the changes were not so straightforward as the cases of homooligonucleotides. It was observed that changes in **r** were affected by neighboring nucleobases. When guanine residue locates in the vicinity of fluorescein, there exists a case that **r** does not change upon hybrid formation (Exp. 6). In order to study the effect of guanine residue on such phenomena, F-ODNs (Type II) were synthesized and their interactions with oligonucleotides were studied (Tab. 2). When they interacted with their complementary oligonucleotides to form hybrids, **r** increased except Exp. 14, where a guanine residue gets close to fluorescein residue upon hybrid formation. Contrarily, **r** in Exp. 16 increased upon hybrid formation, though guanine exists in the vicinity of fluorescein. Stern-Volmer plots using four kinds of nucleosides, showed that guanine drastically and exceptionally quenched the fluorescence of F-ODN (Data not shown). The redox potential of guanine is much different from other three bases. Taking these results into consideration, the phenomena observed in this study might be due to certain fluorescent energy transfer to guanine. Though the detailed mechanism of guanine effects on the fluorescence anisotropy, these results showed that the hybrid formation of oligonucleotides with its complementary strand can be readily studied by simply monitoring fluorescence anisotropy.

These findings were then extended to monitoring the secondary structure of RNA (28). When F-AS hybridized to acceptor sites of RNA, **r** should in-

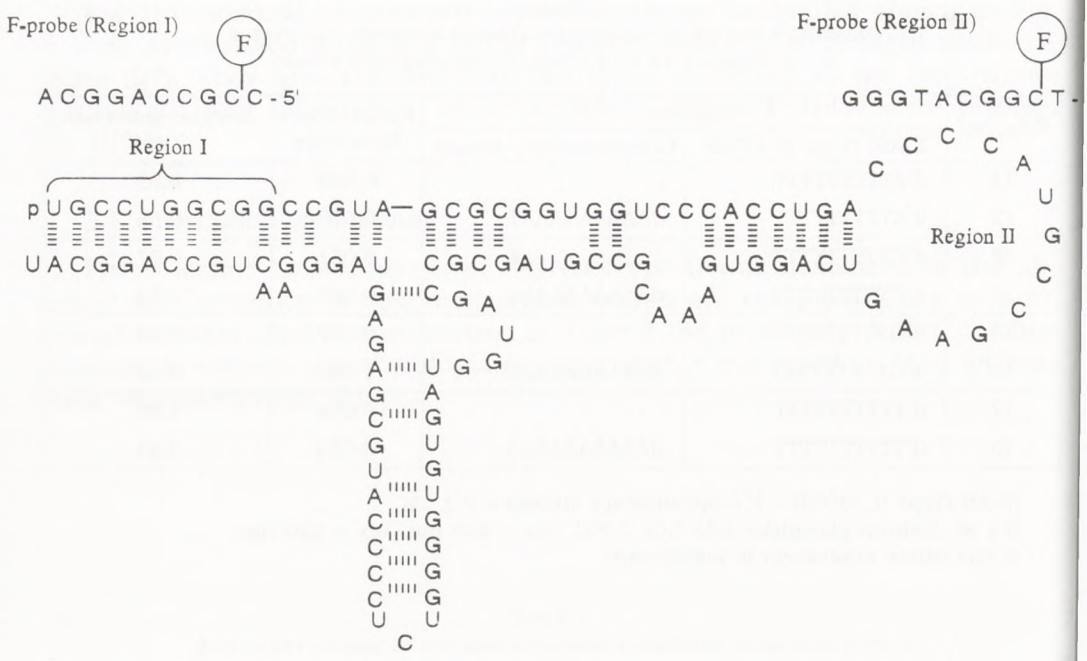

Fig. 2. Proposed secondary structure of *E. Coli* 5S-rRNA (29).

crease. In order to design antisense molecules, the acceptor sites (e.g. loop regions) of RNA should be specified, and, however, it is quite difficult and time consuming works to decide such ternary structure. As a model molecule, 5S-ribosomal RNA (5S-rRNA; from *E. coli*, 120 bs) was chosen and the proposal secondary structure is shown in Figure 2 (29). Anisotropy of F-ODN (Type I) complementary to Region II increased upon mixing with 5S-rRNA, whereas F-ODN (Type I) complementary to Region I did not appreciably changed (Tab. 3). These results show that the F-ODN to Region II formed a hybrid with 5S-rRNA and that, subsequently, Region II is presumably a loop region. Also, as the Region I rejected F-ODN, it is found that the region is a stem one.

From the results, the method showing in this manuscript is found to be applicable to monitor both primary and secondary structures of RNA in the condition which RNA exists in the physiological conditions.

TABLE 3
ANISOTROPY CHANGE OF FLUORESCEIN-LABELED ANTISENSE DNA COMPLEMENTARY TO 5s-rRNA

Exp. No.	Sequence		Fluorescence Anisotropy
	F-AS* (Type I: ODN)	Complementary Strand	
19	dC*CGCCAGGCA		0.026
20	dC*CGCCAGGCA	5s-rRNA	0.027
21	dT*CGGCATGGG		0.037
22	dT*CGGCATGGG	5s-rRNA	0.073

[F-AS (Type I; ODN)] = 0.1 μM, [5s-rRNA] = 0.2 μM,
10 mM Tris-HCl (pH 7.0), 0.1 M. NaCl, 20°C,
λex = 490 nm, λex = 520 nm.
*: site where fluorescein is introduced.

3.2. Interaction with serum proteins

It is well known that serum albumin plays an important role as a drug-carrier, and it has several binding sites with different characteristics (30). Using fluorescence depolarization spectroscopy, interaction of antisense molecules with serum proteins was studied without denaturing these proteins. OPT was first subjected to this study, as OPT is regarded to be most effective among antisense molecules reported. F-OPT (Type II) was mixed with HSA and the consequent anisotropy change was evaluated. As shown in Table 4, the anisotropy of the F-OPT largely increased upon increment of HSA concentration up to 100 μM. As the viscosity change in those systems is negligible in the concentration range studied, changes in anisotropy are considered to be attributed to the interaction of fluorescent materials with HSA. When non-labeled ODN complementary to F-OPT was added to the mixture, anisotropy values decreased to some extent. The changes could be attributed to the dissociation of F-AS=HSA complexes and to consequent hybrid formation between F-OPT and added ODN. When other serum proteins such as HIG and HTF were used instead of HSA, anisotropy values also increased, although the values were much smaller than HSA systems (Fig. 3). These results suggest that F-OPT interact with serum proteins, and the affinities to proteins were largely different among proteins.

The effect of linkage of antisense molecules was then studied. Four kinds of linkages as shown in Figure 1 were chosen. Anisotropy values of all antisense molecules systems increased as the concentration of HSA increased, and the feature of F-OPT system was very characteristic as shown in Fig. 4. For numerical evaluation, the binding constants were calculated from Scatchard plots between F-AS and HSA. Table 5 summarized Kd values of various F-AS systems.

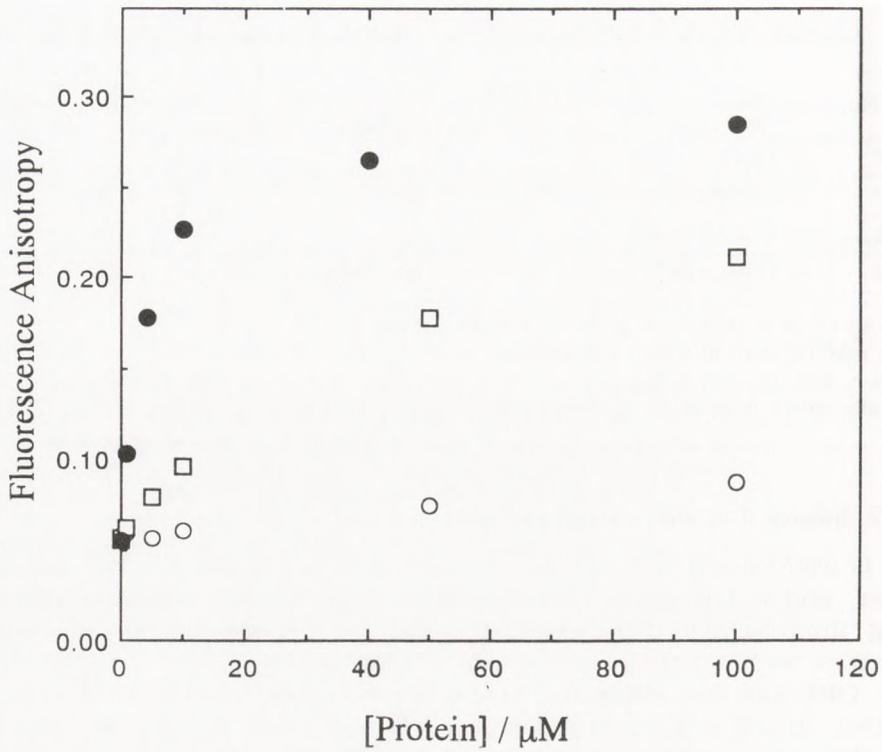

Fig. 3. Isotherm of fluorescence anisotropy of fluorescent-labeled antisense molecules in the presence of human serum proteins.
[F-OPT] = 0.1 μM, λex = 490 nm, λex = 520 nm, band path = 5 nm (exc.& em.).
● : HSA, □ : HIG, O : OHTF

These results suggest that [1] antisense molecules may interact with serum proteins forming a certain complex upon administration to a body, [2] antisense molecules may be easily translocated to other biological substances such as blood cells and genes, [3] OPT interact with HSA more dominantly than other antisense molecules such as ODN, OMP, and PNA, and [4] the binding of F-AS with proteins was sequence-dependent, as shown in Table 2. From these results, it is suggested that we should pay much attention to the "apparent" antisense effect of OPT (31,32). The effect might be sequence-specific and non-antisense ones.

TABLE 4
FLUORESCENCE ANISOTROPY CHANGE OF FLUORESCEIN-LABELED OPT UPON INCREMENT OF HUMAN SERUM ALBUMIN

[HSA] μM	Fluorescence Anisotropy			
	F-AS* (Type II; OPT)			
	r	I_F rel[1]	τ[3]nsec	r (+ ODN)[2]
0	0.055	1.00	4.4	0.063
0.1	0.055	1.11	—	—
1.0	0.103	1.26	5.2	—
4.0	0.178	1.29	—	0.146
6.0	0.210	1.37	—	—
8.0	0.222	1.33	—	0.169
10.0	0.227	1.50	5.0	—
40.0	0.265	1.41	—	0.209
70.0	0.277	1.37	—	—
100	0.285	1.32	4.6	0.250

[F-AS* (Type II; OPT)] = 0.1 μM., 0.1 M Phosphate buffer (pH = 7.0), 20°C, λexc = 490 nm, λem = 520 nm.

[1]relative fluorescence intensity,

[2]oligonucleotide (dGACGATGCCC) complementary to F-ODN and F-OPT, 0.1 μM,

[3]fluorescence life time.

As to the binding mode of F-OPT and F-ODN to HSA, both electronic and hydrophobic interactions might be considered. As the total net charges and the structures were similar between F-OPT and F-ODN, the difference in binding ability is due to the presence of sulfur-contained atoms in internucleotide linkages. It is considered that the electrostatic force of attraction was not the major cause of the binding in the case of F-OPT and F-ODN at physiological conditions. As shown in Table 5, the binding was dependent to the sequence. PNA did not tightly bind to HSA, though those molecules are more hydrophobic than OPT. It is probable that the binding of antisense molecules is attributed to the combined effect of both electronic and hydrophobic interactions.

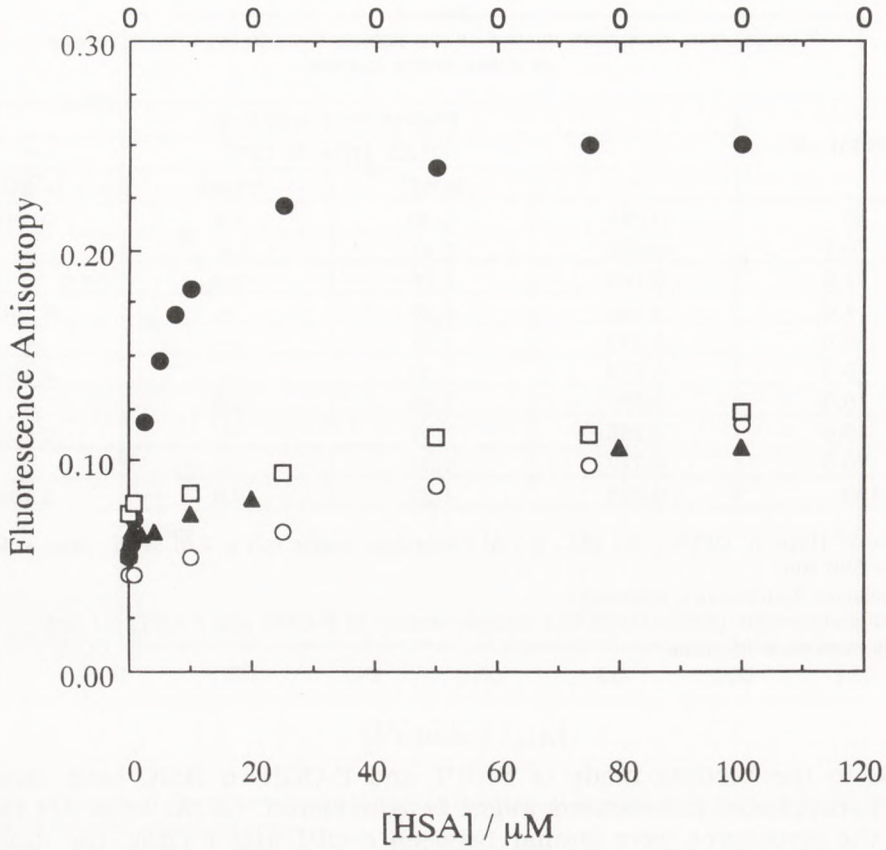

Fig. 4. Isotherm of fluorescence anisotropy of fluorescent-labeled antisense molecules in the presence of human serum albumin.
[F-AS] = 0.1 μM, λex = 490 nm, λex = 520 nm, band path = 5 nm (exc.& em.).
●: F -OPT, ○: F-ODN, △: F-OMP, □: F-PNA

TABLE 5
DISSOCIATION CONSTANTS OF FLUORESCEIN-LABELED ANTISENSE DNAs (TYPE II) TO HUMAN SERUM ALBUMIN

Sequence (Type II)	Dissociation Constant/ x 10^6M			
	Type of Antisense Molecule			
	OPT	ODN	PNA	OMP
dTCCATGCAT	6.1	210	330	
dAGAGGGGCAT	9.0	330	88	
dATGCCCCTCA	7.0	200	280	
dTTTTTTTTTTTTTTT		100		33

[F-OPT] = [F-ODN] = [F-PNA] = [F-OMP] = 0.1 μM, 0.1 M phosphate buffer (pH = 7.0), 20°C, λexc = 490 nm, λem = 520 nm.

Conclusively, results in this paper showed that antisense molecules interact with serum proteins probably in non-sequence specific manners before they reach to the target cells or organs. It was also showed that the extents of the interactions were dependent on kinds of proteins, and that the interactions might be dependent on the sequence of antisense molecules. These results indicate that investigation of the binding characteristics of antisense molecules with cellular proteins as well as with serum proteins would be crucial to clarify the mechanism of antisense effects. For such research, the method adapted in this study is found to be suitable (33) and useful in spite of several problems to be overcome.

Acknowledgments

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Interaction of Antisense Oligonucleotides with DNA and Proteins Studied by Fluorescence Polarization Spectroscopy

Summary

In order to evaluate the antisense effects in biological systems, the interaction of antisense molecules with biological substances such as DNAs and human serum proteins was studied by fluorescence depolarization spectroscopy. When an antisense oligonucleotide anchoring fluorescein (F-AS) was mixed with DNAs or proteins, the anisotropy value changed, indicating that antisense oligonucleotides appreciably interact with DNAs and serum proteins, and that such interaction may play important roles on antisense gene regulation.

Key words:
antisense oligonucleotides, fluorescence depolarization spectroscopy.

Address for correspondence:
Akira Murakami, Department of Polymer Science and Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan, fax: 0-081-75-722-2938.