

Selected Paper

Cholesterol-Appended β-(1→3)-D-Glucan Schizophyllan for Antisense Oligonucleotides Delivery to Enhance the Cellular Uptake#

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Schizophyllan is a natural β-(1→3)-D-glucan existing as a triple helix in water and as a single chain in dimethyl sulfoxide (DMSO), respectively. As we already reported, when a homo-polynucleotide is added to a schizophyllan solution, the single chain of schizophyllan forms a complex with the polynucleotide. One of the potential applications of this novel complex is an antisense-oligonucleotide (AS ODN) carrier. The present paper describes a modification technique that enables us to introduce a cholesterol group only to the side chain of schizophyllan. We prepared four cholesterol-appended schizophyllans with different modification levels. Using these compounds, we made complexes and carried out an in vitro antisense assay, administrating a phosphorothioate AS ODN to the several cell lines to depress their c-myb mRNA. When we used 2.2–2.3 mol% modified schizophyllan as the carrier, the antisense effect was most enhanced among others. The addition of β-cyclodextrin improved the complexation ability as well as the up-take for highly modified samples. Furthermore, the cytotoxicity for these modified schizophyllan samples was negligibly as small as the natural (unmodified) schizophyllan. The present work has thus clarified that schizophyllan can act as a new potential candidate for AS ODN carriers.

Antisense oligonucleotides (AS ODNs), designed to suppress a particular gene expression, have considerable potential as a research tool as well as a therapeutic agent.[1,2] The major advantage of this strategy over the conventional drugs is related to the specificity of the action due to silencing a particular mRNA to down-regulate the protein expression. However, there are two major issues to overcome, namely, the instability of AS ODNs in biological fluids and the low uptake efficiency into the target cells.[3] The instability of AS ODNs is mainly ascribed to hydrolysis mediated by deoxyribonucleases. To provide resistance against deoxyribonucleases, various oligonucleotide analogs have been developed, of which the phosphorothioate analogs have been widely used, and some of them entered clinical trial.[4]

Although phosphorothioate AS ODNs have great advantage over phosphodiester AS ODNs, there are some serious drawbacks for practical use. Among them, phosphorothioate AS ODNs tend to bind to serum proteins, such as albumin, in a non-specific manner, leading to an unfavorable no-antisense effect in biological systems. When phosphorothioate AS ODN forms a complex with a polysaccharide schizophyllan (described below in more detail), and then added to a cell–culture medium containing albumin, the albumin–protein association is reduced, and thus the antisense effect is enhanced.[5] This study suggests that the complexation of phosphorothioate AS ODN can reduce the no-antisense effect.

In order to increase the cellular up-take of AS ODNs, cholesterol has been attached directly to the oligonucleotide.[6–8] It was found that the cholesterol conjugated AS ODN is ingested through the LDL receptors (for phagocytic cells, scavenger receptors are also responsible for the ingestion). One of the advantages of using cholesterol is specific delivery to Kupper cells.[7] However, once cholesterol is chemically attached to AS ODN, release of the AS ODN from endosome may become difficult. This is because the hydrophobic cholesterol moiety tends to anchor inside of lipid bilayers. Polyethylenimine can form a complex with ODN as well as plasmid DNA, and performs endosomal release by osmotic swelling due to a proton sponge effect.[9] Kim et al.[10,11] introduced cholesterol to polyethylenimine to dramatically improve the cellular uptake of plasmid DNA. For AS ODN, however, a compaction problem has been pointed out; that is, the cationic polymer is bound to anionic ODN too tightly to be released even when necessary.[3] Furthermore, the cationic property of the polyethylenimine/AS ODN complex should eliminate cell specificity, because the regular pinocytosis ingests the plasma membrane together with cations that bind to the anionic membrane surface. It seems, therefore, that some polymeric material, which can bind to AS ODN without using electrostatic interactions, should overcome the compaction and non-specificity problems.

Sakurai and Shinkai[12,13] found that a β-(1→3)-D-glucan

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schizophyllan can form a complex with some polynucleotides, and the complex is applicable to AS ODN carriers.[5] Schizophyllan is an extracellular polysaccharide produced by the fungus *Schizophyllan commune*; the main chain consists of β-(1→3)-D-glucan and one β-(1→6)-D-glycosyl side chain links to the main chain at every three glucose residues [see: Fig. 1a for the chemical structure].[14] Schizophyllan adopts a triple helical conformation in water and a random coil in dimethyl sulfoxide (DMSO).[15–18] When water is added to the DMSO solution (renaturation), the triple helical structure can be partially retrieved through this process, although the entire chain structure is not the same as that of the original triple helix.[19,20] The single chain of schizophyllan (s-SPG) forms a macromolecular complex with some homo-phosphodiester polynucleotides [such as poly(C), poly(A), poly(U), poly(dA), and poly(T)], when the polynucleotide is present in the renaturation process.[12,13] As a matter of fact, other β-(1→3)-D-glucans, such as curdlan and lentinan, can also form the same kind of complexes as schizophyllan; therefore, this complexation is a general property for β-(1→3)-D-glucans.[21,22] Some of the novel features for this complex are: (1) the complex is remarkably stable and considerably water-soluble in the physiological conditions,[12,13] (2) complexation occurs in a highly stoichiometrical manner and the stoichiometric number indicates that two schizophyllan units and three base units interact with each other [see: Fig. 1b],[12,13] (3) the complex is automatically dissociated when the pH becomes less than 6.0, because protonation of the nucleotide base induces conformational changes, which causes dissociation of the complex.

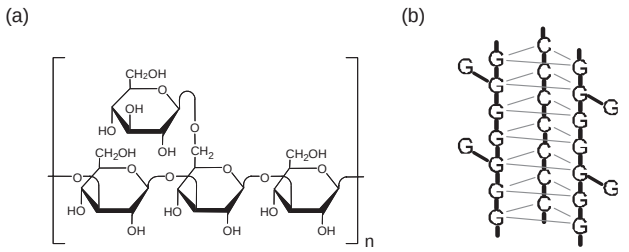

Fig. 1. Repeating units of schizophyllan: (a) and a schematic illustration of the complex: (b). In (b), the capital letters of G and C represent the glucose and base, and the gray lines shows the hydrogen bonds, respectively.

When schizophyllan is used as an AS ODN carrier, the three features of (1)–(3) mentioned above seem to have great advantage.[23,24] Especially, for phosphorothioate AS ODN, the above-mentioned (3) feature enables the complex to release the bound AS ODN in endosome when the pH is lowered, and the free AS ODN may diffuse to cytosol. In the present work, we have made attempts to modify s-SPG with cholesterol (see: Fig. 2) to enhance the cellular uptake and to carry out an in vitro assay to evaluate the ability as AS ODN carriers.

Results and Discussion

Complexation between AS ODNs and Chol(X)-SPG. Figure 3 compares the gel electrophoresis migration patterns for naked AS-c-myb (lane 1), s-SPG + AS-c-myb (lane 2), and four mixtures made from AS-c-myb and each Chol(X)-SPG (lanes 3, 4, 5, and 6), where "X" in Chol(X) indicate the modification level of cholesterol (see: Table 1). We already knew that AS-c-myb and s-SPG form a complex and the resultant complex stays at the starting point.[5,13] A small amount of free AS-c-myb has been observed in lane 2, probably, which was dissociated by applying an electric field, and the image-amplification process merely made it visible. In this experiment, we prepared the complex in an excess amount of s-SPG over the stoichiometry ($M_{\text{s-SPG}}/M_{\text{ODN}} = 2/3$) and, in fact, spectroscopic measurements with UV and CD confirmed that all AS-c-myb molecules were incorporated into the s-SPG + AS-c-myb complex.[5] The migration patterns for the mixtures of Chol(1)-SPG, Chol(2)-SPG, and Chol(3)-SPG are similar to that of the s-SPG/AS-c-myb complex, indicating that the Chol(X)-SPG/AS-c-myb complex is formed in each mixture, and little free AS-c-myb remains. However, the Chol(4)-SPG + AS-c-myb mixture shows a different pattern from others, dissociating more free AS-c-myb from the complex than the other mixtures. This feature suggests that the complexation ability of Chol-SPG(4) is not as good as the others.

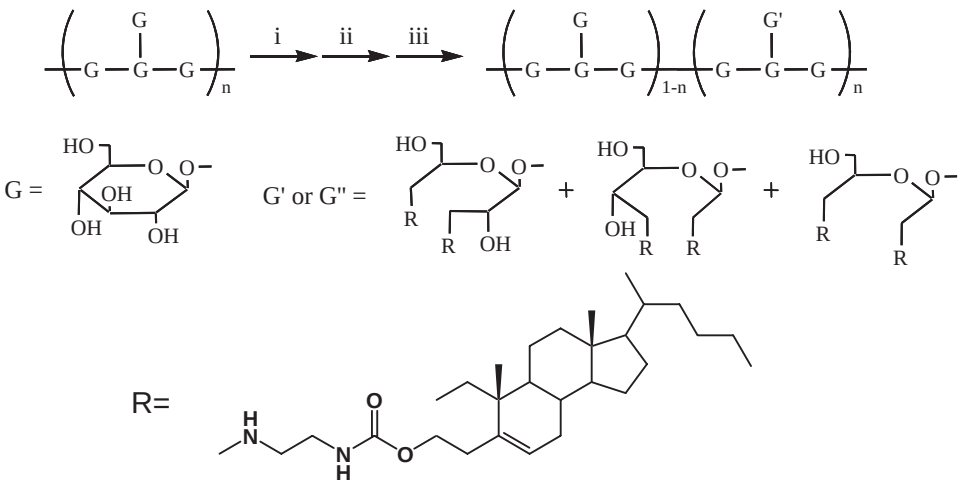

Fig. 2. Reaction scheme to introduce the cholesterol moiety into the side chain of s-SPG. (i) Oxidation of the side chain of SPG with $NaIO_4$ in H_2O at 4 °C for 2 days, (ii) reaction with 3β-cholest-5-en-3-yl *N*-(2-aminoethyl)carbamate in DMSO at room temperature for 2 days, (iii) reduction with $NaBH_4$ in DMSO at room temperature for 1 day.

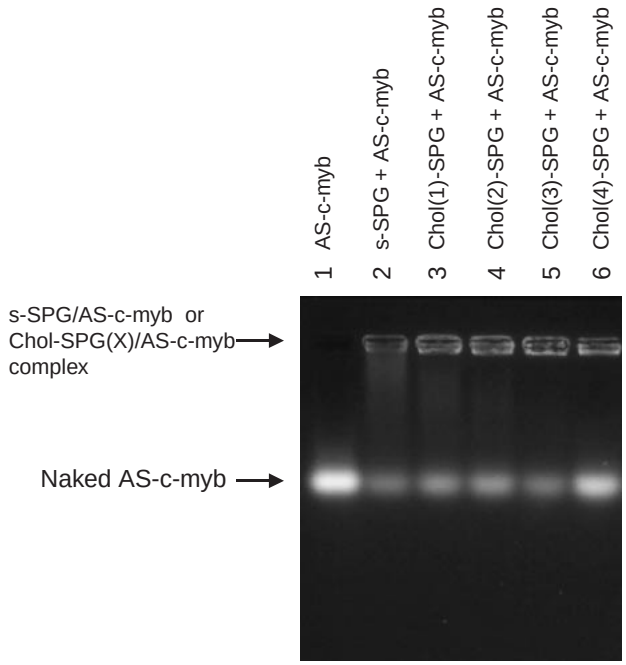

Fig. 3. Comparison of the gel electrophoresis patterns among AS-c-myb (lane 1), s-SPG + AS-c-myb (lane 2), and four mixtures made from AS-c-myb and each Chol(X)-SPG (lanes 3, 4, 5, and 6). 2 wt % NuSieve agarose gel (BMA) was used and the gel was stained with GelStar® (BMA). When the complex is formed, the migration does not occur and the complex stays at the starting hole. The composition ratio of the mixture is an excess amount of the SPG samples over the stoichiometry.

Table 1. Modification Level of Cholesterol and Sample Codes

Sample Codes	Modification level[a)]/mol %
s-SPG	0
Chol(1)-SPG	1.9
Chol(2)-SPG	2.2
Chol(3)-SPG	2.4
Chol(4)-SPG	2.8

a) The modification level was estimated by titration with 6-(*N*-dansyl-D-leucylamino)-6-deoxy-β-cyclodextrin.

Poly(C) exhibits a dramatic increment in the CD spectrum upon complexation with s-SPG, due to base stacking.[13] Figures 4A and B show the CD spectra for naked poly(C): curve (a), s-SPG/poly(C) complex: curve (b), Chol(1)-SPG + poly(C) mixture: curve (c), Chol(3)-SPG + poly(C) mixture: curve (d), and Chol(4)-SPG + poly(C) mixture: curve (e), respectively. The spectra (b) and (c) are almost identical, providing evidence that all poly(C) molecules in the mixture are incorporated in the Chol(1)-SPG/poly(C) complex, and the complex structure is the same as that of the s-SPG/poly(C) complex. On the other hand, with increasing the modification level (from Chol(3)-SPG to Chol(4)-SPG), the CD intensity decreases and the spectral shape becomes similar to that of the naked poly(C). Estimating from the spectral difference, less than 10% of the cytidine bases are bound to the complex in the poly(C) + Chol(4)-SPG mixture, and about 50% of them are bound to the poly(C) + Chol(3)-SPG mixture.

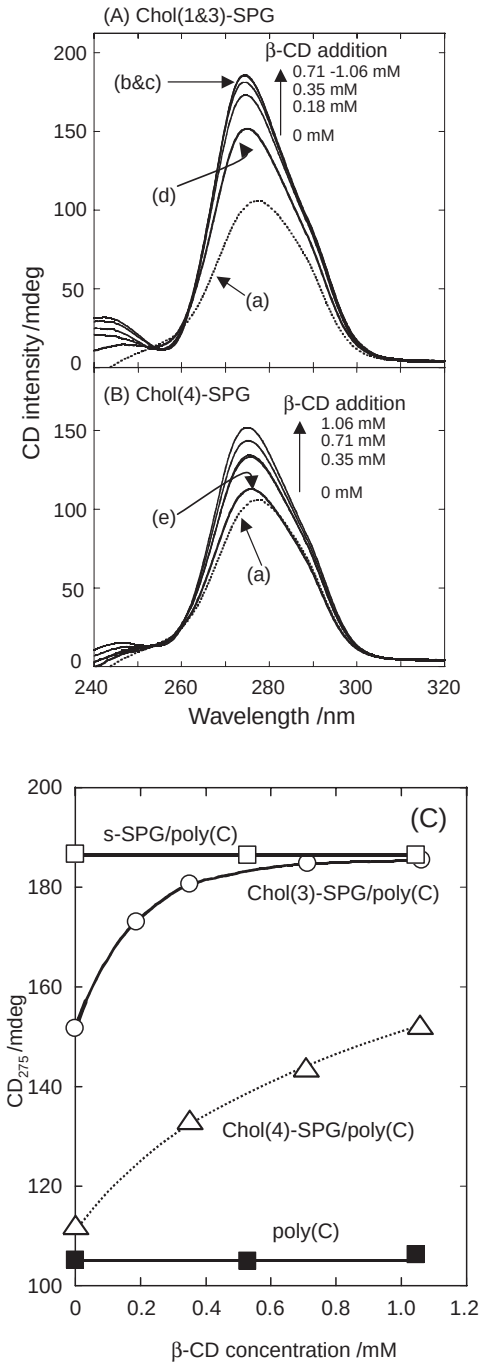

Fig. 4. Comparison of circular dichroic (CD) spectra among naked poly(C): curve (a), s-SPG/poly(C) complex: curve (b), Chol(1)-SPG + poly(C) mixture: curve (c), Chol(3)-SPG + poly(C) mixture: curve (d), and Chol(4)-SPG + poly(C) mixture: curve (e), respectively, and the spectral change upon addition of β-cyclodextrin to poly(C) + Chol(3)-SPG mixture in (A) and poly(C) + Chol(4)-SPG mixture in (B). The panel (C) plots the CD value at 278 nm against the β-cyclodextrin concentration.

There seems to be a discrepancy between the CD and electrophoresis results; the CD shows that the complex is minority

in the poly(C) + Chol(4)-SPG mixture, while electrophoresis suggests the opposite result. The CD spectral change indicates how many bases are free from complexation with SPG, and dose not show how many polynucleotides are free from complexation. Even when a few bases in the polynucleotide are bound to SPG, it may be judged as a complex by an electrophoresis analysis, while (the) CD spectroscopy should show uncompleted complexation, such as the spectra of (c) and (d). This should be the reason for the discrepancy between the two methods.

The above results conclude that the complexation ability decreases with increasing the cholesterol modification level. Akiyoshi et al.,[25–27] who introduced cholesterol into pullulan (hydrophilic polysaccharide), showed that the hydrophobic cholesterol moiety can serve as a junction point into aqueous solutions to produce a hydrogel. Although not a polysaccharide, the introduction of cholesterol to poly(L-lysine) drastically changes the polymer conformation.[26] Since a similar effect is expected to occur in our case, the cholesterol attached SPG chain tends to stick together, resulting in interference with the SPG conformation to make it difficult for SPG to form a complex with polynucleotides.

β-Cyclodextrin forms an inclusion complex with cholesterol and the resultant complex is generally soluble to water. The addition of β-cyclodextrin to cholesterol-appended pullulan induces a confrontational change due to alternating the hydrophilic vs hydrophobic balance.[27] Figure 4 also shows how the addition of β-cyclodextrin improves the complexation ability for the Chol(3)-SPG + poly(C) (upper) and Chol(4)-SPG + poly(C) (lower) mixtures. For Chol(3)-SPG, the addition drastically develops the CD spectrum; it became almost identical with the s-SPG/poly(C) complex when we added 1.0 mM β-cyclodextrin. On the other hand, even when we added the same amount of β-cyclodextrin to the Chol(4)-SPG + poly(C) mixtures, the CD spectrum did not recover completely. Panel (c) in Fig. 4 plots the CD value at $\lambda = 278$ nm against the β-cyclodextrin concentration. The addition of β-cyclodextrin does not cause any change for both naked poly(C) and s-SPG/poly(C) solutions. For the Chol(3)-SPG + poly(C) mixture, the CD increases, and seemingly merges into the complex curve at 0.8 mM or more. When β-cyclodextrin is added to the Chol(4)-SPG + poly(C) mixture, the CD values increase by almost 50% at the highest addition.

To conclude this section, the Chol(1)-SPG + ODN mixture only contains the Chol(1)-SPG/ODN complex, while the other Chol(X)-SPG mixtures consist of the complexed and naked ODN. When β-cyclodextrin is added to Chol(2)-SPG + ODN and Chol(3)-SPG + ODN mixtures, most ODN is incorporated in the complex at 1 mM, or more. However, some naked ODN remains, even when much β-cyclodextrin is added to Chol(4)-SPG + ODN.

Cellular Ingestion of Chol(1)-SPG/ODN Complex. To examine whether Chol(1)-SPG enhances the cellular uptake of AS ODN, we exposed A375 cells to a complex made of TR-Chol(1)-SPG [Texas Red®-labeled Chol(1)-SPG] and 5′-FITC-labeled (dA_{50}) oligonucleotide, and observed the cell morphology, comparing with that of the Texas Red®-labeled s-SPG complex (without cholesterol). Figure 5 shows typical confocal microscopic images. The photo (f) shows many bright-green spots (FITC labeled ODN) in the cell, while such bright spots are not observed very much in photo (b). Comparing (c) and (g), the red color (TR labeled SPG) is more condensed in the Chol(1)-SPG complex than in the s-SPG complex. All of the results indicate that cholesterol enhames the up-take of ODN. It is interesting that there is a clear difference in the distribution of Texas Red®-labeled SPG between (c) and (g). In panel (g), the red color is deeper than that of panel (c), and Chol(1)-SPG seems to be distributed more spottily than

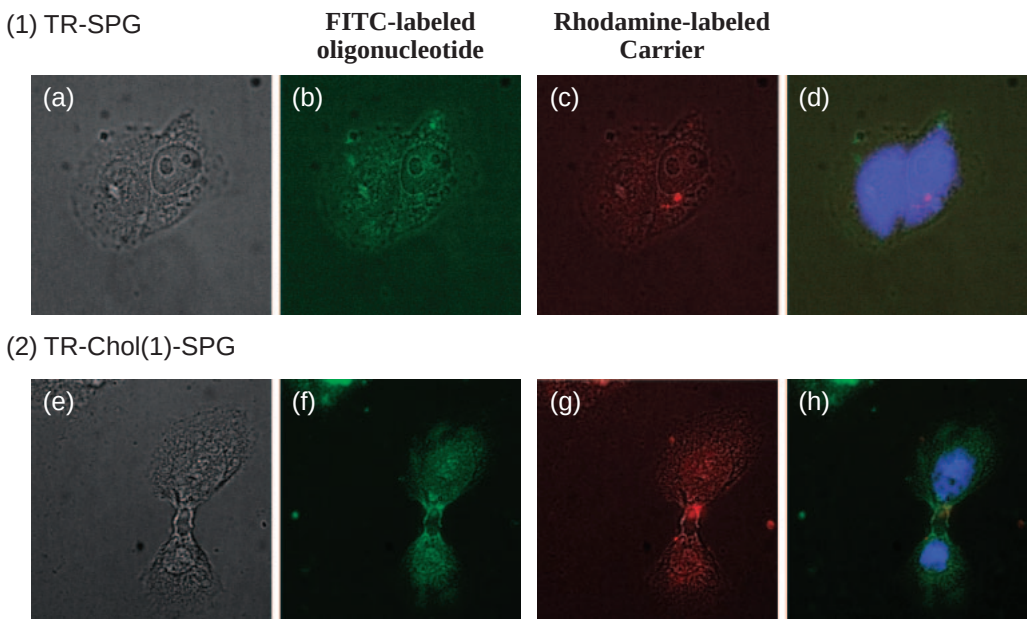

Fig. 5. Distribution of FITC labeled ODN and Rodamine labeled SPG in A375 measured with confocal fluorescent microscopy, comparing between the cholesterol modified SPG (2) and unmodified one (1). The photos (b) and (f) are FITC fluorescence, (c) and (g) are corresponding Rodamime fluorescence, and (d) and (h) are an image stained with DAPI.

that of s-SPG. This echymotic distribution can be evidence that Chol(1)-SPG is located in the vesicles more than in cytosol. This feature may be ascribed to the endocytosis ingestion of Chol(1)-SPG.

Compared with the difference between (c) and (g), the difference in green color between (b) and (f) is less prominent. This means that when we exposed the unmodified SPG complex, the Texas Red®-labeled SPG is hardly ingested, while the FITC-labeled dA_{50} phosphorothioate can be up-taken in some amount, more than expected from the poor ingestion ability of s-SPG. This result suggests that dA_{50} phosphorothioate, itself, is ingested. A pathway has been known to uptake phosphorothioates on the cell surface;[30] therefore, dA_{50} phosphorothioate may be internalized with this path and s-SPG might be dissociated during this process.

Comparison of the Carrier Efficiency for Antisense ODN Delivery. Figure 6a shows A375 cell growth after being cultured for three days when AS-c-myb was dosed in 25 and 50 μg/mL, comparing among naked AS-c-myb, s-SPG/AS-c-myb, and four Chol(X)-SPG + AS-c-myb mixtures. Here, the cell growth is normalized by the cell number for the control. Figures 6b and 6c present the growth when S-c-myb and MS-c-myb were exposed under the same conditions as the AS-c-myb assays. The naked AS-c-myb decreases the growth by 25% at 25 μg/mL dose and 45% at 50 μg/mL dose. On the other hand, when the cells were exposed to the G-rich mismatch sequence (MS-c-myb), the growth was more reduced than that of the corresponding sense sequence (S-c-myb). This can be explained by an anti-proliferation activity (or cytotoxicity) due to a non-antisense effect for the contiguous G residues.[31] This G domain cytotoxic effect is estimated to decrease the growth by 10% for a 25 μg/mL dose and 20% for a 50 μg/mL dose, respectively. Therefore, comparison between the panels (a) and (c) clarifies that the antisense effect overwhelms the G domain effect in the present assay.

In panel (a), s-SPG/AS-c-myb decreases more efficiently than naked doses. When we carried out the T-test for those two groups (naked AS-c-myb and s-SPG/AS-c-myb) at a 25 μg/mL dose, the T-value was calculated to be 6.0, and thus we can consider that this difference between the two groups is statistically significant. When AS-c-myb was added as a complex with Chol(1)-SPG, the growth was dramatically reduced to 63% at a 25 μg/mL dose and to 46% at a 50 μg/mL dose. By comparing the corresponding S-c-myb and MS-c-myb assays, it is clear that cholesterol enhances the antisense effect. When we increased the cholesterol content from Chol(1) to Chol(3)-SPG, the antisense effect was increased. However, with further increment of the cholesterol content (i.e., from Chol(3) to Chol(4)-SPG), the antisense effect was decreased (the growth is increased). For Chol(4)-SPG + AS-c-myb, the growth was essentially the same as that of the naked assay (the T value is less than 1.0). This feature can be explained by the fact that Chol(4)-SPG is poor at complexing with ODNs, as shown in the above section. When octa-arginine or arginine–glycine–aspartic acid tripeptide was attached to the s-SPG chain, and the resultant SPG/AS-c-myb complex was exposed to A375 cells, the growth was reduced to 20–40%.[24] The enhancement of the antisense effect of Chol(1)-SPG + AS-c-myb is comparable to those functional peptide appended SPG.

The enhancement of the antisense effect shown in Fig. 6 supports that the complexed AS-c-myb specifically depresses the c-myb protein expression in the mRNA level. To confirm this, we carried out an RT-PCR assay, comparing between

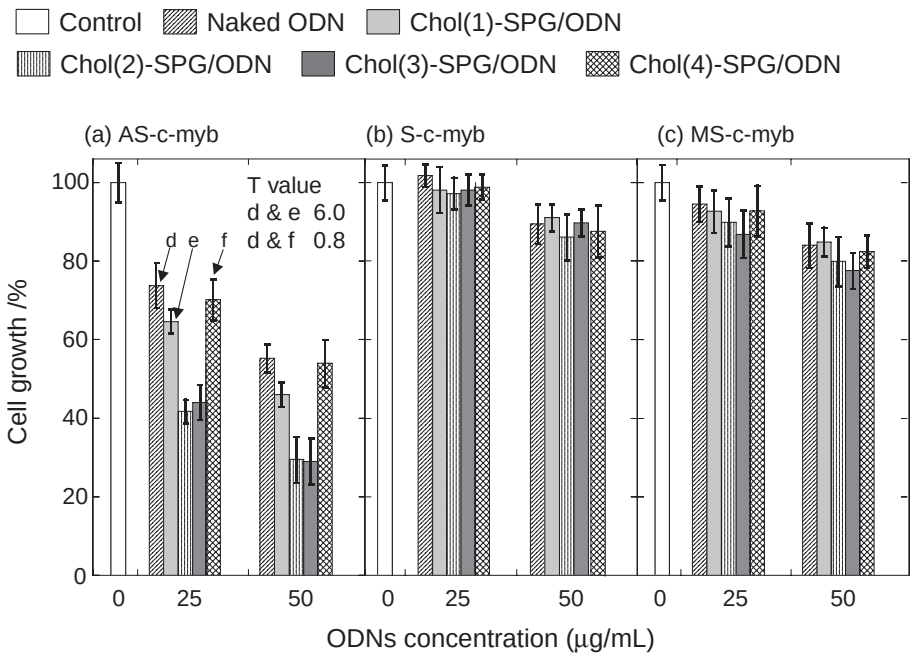

Fig. 6. Comparison of the cell growth when the A375 cell lines were exposed to AS-c-myb and its complex with s-SPG or Chol(X)-SPG in (a), to S-c-myb and its complex with s-SPG or Chol(X)-SPG in (b), and MS-c-myb and its complex with s-SPG or Chol(X)-SPG in (c). The cell numbers were determined with Cell Counting Kit-8® (Dojindo, Japan), incubated the cells for three days. The s-SPG:AS-c-myb molar ratio was fixed at 1:1.

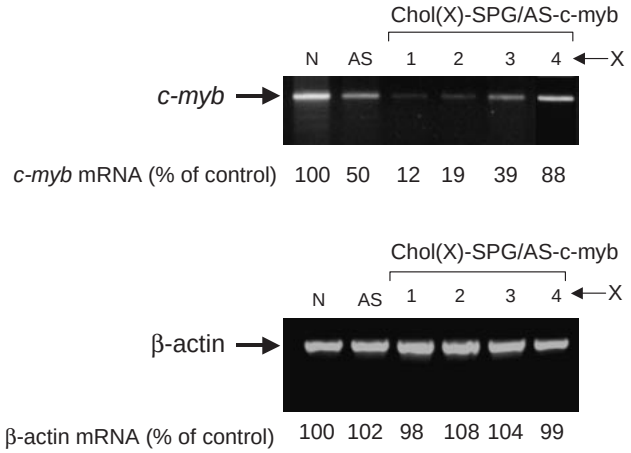

Fig. 7. Sequence specific mRNA silencing confirmed by RT-PCR (reverse-transcriptase-mediated PCR), comparing between naked ODN and its complex. A375 was treated with 60 μg/mL of AS ODN (targeting c-myb mRNA) or its Chol(X)-SPG complexes. PCR products were resolved by agarose gel electrophoresis and visualized by GelStar®. Amplified DNA fragments were quantified by a gel documentation system (Alpha DigiDOC gel documentation system, Alpha Innotech). The numbers under the gel image indicate the relative intensity of the mRNA band to the reference.

c-myb and β-actin mRNAs, when we dosed naked AS-c-myb or each one of four Chol(X)-SPG + AS-c-myb mixtures. The results are presented in Fig. 7. As shown in the lower panel, there is no difference in the amount of β-actin mRNA. However, for c-myb mRNA (the upper panel in the figure), the Chol(1)-SPG/AS-c-myb decrease the amount to 12%, while the naked assay shows 50%. With increasing the cholesterol modification, the mRNA amount increases, and finally reaches 88% for Chol(4)-SPG. This feature is consistent with the cell growth presented in Fig. 6. Good agreement between both results is strong evidence that AS-c-myb has silenced the target mRNA in a sequence-specific manner and the cholesterol-appended complex delivers the ODN efficiently.

Table 2 summarizes the cell growth at 50 dose after 3 days for all cell several lines: Colo205 (colon cancer), LS174T (rectal cancer), HepG2 (hepatoma), and A375 (melanoma). Although more investigation is necessary, there is an appreciable difference between the cells for enhancing of the antisense effect. For example, the growth is decreased from 73% (naked AS-c-myb) to 35% (Chol(1)-SPG/AS-c-myb) for HepG2, while it is from 55% to 31% for Colo205. It seems that rectal and hepatic cancers respond more sensitively than others.

Addition of β-Cyclodextrin to Chol(X)-SPG + AS-c-myb and Its Antisense Assay. Figure 8 compares the cell growth when β-cyclodextrin was added to the assay system. Here, the Colo205 and LS174T cell lines were used. The addition does not create any change in the cell growth for the control, naked AS-c-myb and s-SPG/AS-c-myb, indicating no direct influence from β-cyclodextrin on cell proliferation. When β-cyclodextrin was added to the Chol(X)-SPG + AS-c-myb mixtures, the growth decreased when more β-cyclodextrin was added. This should be ascribed to an enhancement of the antisense effect, and the more prominent effect observed for the more cholesterol-appended SPG (compare Chol(1)-SPG + AS-c-myb and Chol(4)-SPG + AS-c-myb). At 8 mM β-cyclodextrin, the growth was reduced to no more than 20% for Chol(4)-SPG + AS-c-myb, which is lower than Chol(1)-SPG + AS-c-myb and less than half of the case when no β-cyclodextrin was added to Chol(4)-SPG + AS-c-myb. As shown by CD and electrophoresis measurements, Chol(4)-SPG and Chol(3)-SPG have poor ability to bind to AS-c-myb, and thus many free AS-c-myb should be present in the mixtures. The addition of β-cyclodextrin improves the complexation ability due to encapsulating the cholesterol moiety. It is interesting that the cholesterol bound to β-cyclodextrin does not chance the ingestion ability. This feature can be explained as follows. The host/guest interaction between β-cyclodextrin and cholesterol is reversible, although the major component at equilibrium is the complex. Therefore, the encapsulated cholesterol cannot interfere with the complexation between SPG and AS-c-myb. However, once the cholesterol binds to the recognition site on the cell surface, the equilibrium

Table 2. Numerical Comparison of the Cell Growth among the Cholesterol-Appended Carriers

Samples	Cell growth/%			
	Colo205	LS174T	HepG2	A375
Control	100.0 ± 4.7	100.0 ± 4.3	100.0 ± 4.6	100.0 ± 5.2
AS-c-myb	55.2 ± 5.8	51.4 ± 3.1	73.3 ± 5.1	52.8 ± 4.9
s-SPG/AS-c-myb	46.3 ± 4.9	42.4 ± 2.9	64.6 ± 4.2	43.1 ± 2.4
Chol(1)-SPG/AS-c-myb	30.7 ± 5.1	26.6 ± 3.6	35.2 ± 5.7	31.7 ± 3.1
Chol(2)-SPG/AS-c-myb	28.9 ± 5.8	30.7 ± 5.6	34.1 ± 3.8	33.2 ± 5.5
Chol(3)-SPG/AS-c-myb	34.7 ± 3.2	40.0 ± 4.8	49.5 ± 5.1	38.4 ± 4.4
Chol(4)-SPG/AS-c-myb	54.6 ± 6.2	53.3 ± 4.8	61.4 ± 6.1	50.3 ± 6.0
S-c-myb	89.4 ± 5.0	87.3 ± 3.7	96.7 ± 3.3	88.0 ± 6.5
s-SPG/S-c-myb	91.1 ± 3.4	87.9 ± 2.6	96.6 ± 4.7	88.1 ± 3.6
Chol(1)-SPG/S-c-myb	86.1 ± 5.8	85.6 ± 4.6	97.0 ± 6.0	85.2 ± 5.8
Chol(2)-SPG/S-c-myb	89.7 ± 3.4	88.5 ± 2.1	95.3 ± 6.3	89.2 ± 3.2
Chol(3)-SPG/S-c-myb	90.8 ± 6.1	85.4 ± 6.6	98.1 ± 2.2	87.2 ± 5.3
Chol(4)-SPG/S-c-myb	87.9 ± 6.6	88.9 ± 5.2	94.2 ± 5.1	85.4 ± 4.6

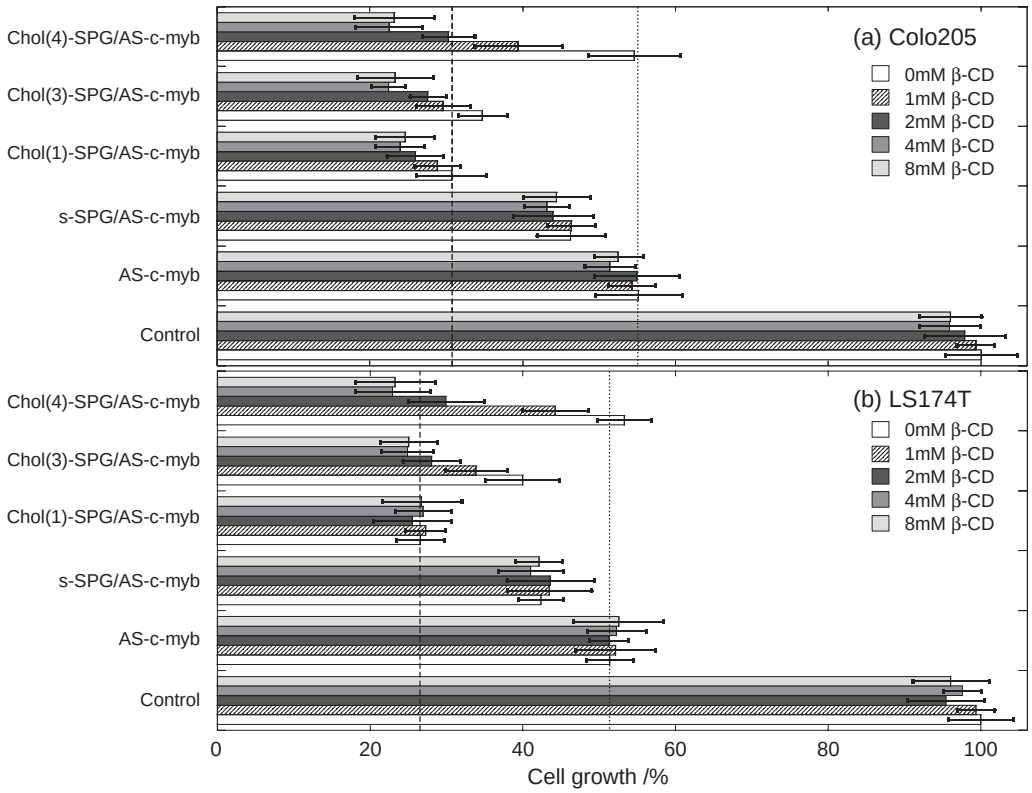

Fig. 8. β-Cyclodextrin addition effect on the cell growth for Colo205 and LS174T, comparing among naked AS-c-myb and its complex with s-SPG or Chol(X)-SPG.

must shift toward the cholesterol vs cell interactions, because of the more favorable interaction than the β-cyclodextrin and cholesterol ones.

Cellular Ingestion Mechanism. Kim et al.[11] delivered plasmid DNA to B16-F0 murine melanoma or Reca murine renal cancer cell by using a polyethylenimine–cholesterol conjugate. When they saturated the LDL receptor with an antibody, and subsequently exposed the polyethylenimine–cholesterol/DNA complex, the corresponding protein expression was decreased. Their study shows that the cholesterol conjugate is ingested through the LDL receptor. Another work showed that the cholesterol-attached ODN is easily associated with LDL in serum, suggesting that it is also up-taken by the LDL receptor.[7] On the other hand, Bijsterbosch et al.[6] carried out an in vivo assay, and showed that 83% of the cholesterol-attached ODN was accumulated in the liver, and that scavenger receptors seemed to be responsible for the cellular up-take. Generally, scavenger receptors recognize certain anionic polymers and also acetylated low-density lipoproteins, and are localized in phagocytic cells. Therefore, it is reasonable that the cholesterol–ODN conjugate was up-taken by the liver cells because this conjugate is negatively charged.

Our Chol(X)-SPG/AS-c-myb is negatively charged, being similar to cholesterol–ODN conjugates. However, since the cell lines used in this work should not have scavenger receptors, our complex may be ingested by same other pathway than scavenger receptors. Figure 9 shows the endocytosis inhibition results. Energy expenditure-type ingestions, such as receptor-mediated endocytosis, are generally reduced with lowering the temperate. Furthermore, wortmannin can specifically inhibit phosphatidylinosite 3-kinase, which is an essential enzyme for receptor-mediated endocytosis.[32] In Fig. 9, both treatments dramatically increase the growth, indicating that the Chol(X)-SPG/AS-c-myb complex is ingested with receptor-mediated endocytosis, probably with the LDL receptor.

Cytotoxicity of the Cholesterol-Appended SPG. Table 3 compares the cell growth when each carrier, itself, was exposed to the cells, while examining the cytotoxicity. As expected from previous work,[24] the cholesterol-modified SPG samples have little cytotoxicity as well as the un-modified SPG. This is in contrast to the fact that Lipofectin itself decreases the growth to 83%, indicating its higher cytotoxic behavior.

Conclusion

We introduced the cholesterol moiety specifically into the schizophyllan side chain by using a specific method, consisting of periodate oxidation of the glucose side chain, and a subsequent reaction between 3β-cholest-5-en-3-yl *N*-(2-aminoethyl) and the formyl terminate, followed by reduction with $NaBH_4$. Using these compounds, we made a complex with AS ODN, and carried out an in vitro antisense assay for the Colo205, LS174T, HepG2, and A375 cell lines to depress their c-myb mRNA. When we used 2.2–2.3 mol % modified schizophyllan as the carrier, the antisense effect was most enhanced among others, which is comparable to those for the functional peptide (such as RGD or octa Arg) appended SPG. Both wortmannin addition and 5 °C culturing depressed the antisense effect, and thus decreased the cellular up-take, indicating that receptor-mediated endocytosis is responsible for up-taking the

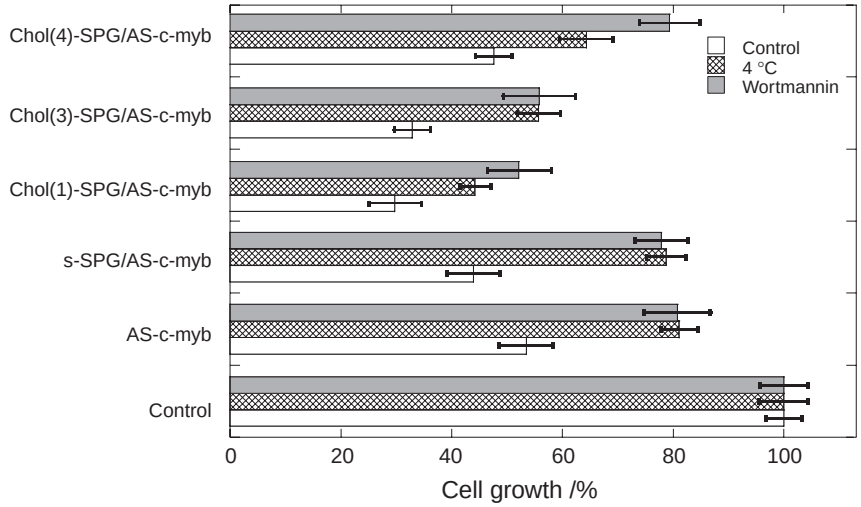

Fig. 9. Endocytosis inhibition assay, comparing among naked AS-c-myb and its complex with s-SPG or Chol(X)-SPG.

Table 3. Cytotoxicity of the Carries Themselves

	Cell growth/%			
Samples	Colo205	LS174T	HepG2	A375
Control	100.0 ± 4.7	100.0 ± 3.7	100.0 ± 4.6	100.0 ± 5.2
s-SPG	95.6 ± 3.1	97.3 ± 4.3	97.2 ± 3.1	99.5 ± 5.5
Chol(1)-SPG	95.1 ± 2.9	98.9 ± 6.2	96.6 ± 4.3	103.2 ± 5.0
Chol(2)-SPG	94.5 ± 3.6	96.6 ± 3.2	94.7 ± 5.7	101.2 ± 6.3
Chol(3)-SPG	93.7 ± 4.8	99.3 ± 5.8	97.5 ± 6.5	97.8 ± 2.7
Chol(4)-SPG	93.3 ± 4.8	96.8 ± 6.2	100.6 ± 3.7	98.6 ± 4.3
Lipofectin				83.0 ± 2.0

cholesterol-appended schizophyllan. The addition of β-cyclodextrin improved the complexation ability as well as the uptake for highly modified samples. The present work has thus clarified that schizophyllan can act as a new candidate for efficient and non-cytotoxic AS ODN carriers.

Experimental

Materials and Cells. Taito Co., Ltd., (Japan) kindly supplied the schizophyllan sample. The weight-average molecular weight (M_w) and the number of repeating units were found to be 1.5×10^5 and 231, respectively.[13] The ODN sequence of 5′-GTGCCGGGGTCTTCGGGC-3′ is well-known to bind to c-myb mRNA, and lead to the depression of c-myb.[30,31] However, since short and hetero ODNs cannot bind to s-SPG, we had to attach a poly(dA) tail with 40 bases to the 3′ ends of this sequence. Thus, in this study, 5′-GTGCCGGGGTCTTCGGGC-$(dA)_{40}$-3′ phosphorothioate was used as an AS ODN, and denoted by AS-c-myb. As a negative control, we used two ODNs, containing the sense sequence, 5′-CACGGCCCCAGAAGCCCG-3′, and a mismatch sequence of AS-c-myb, 5′-GTC CTG GGG TCG TCG GGC-3′[30] (the mismatched bases are underlined). For the same reason, the two sequences were connected to the $(dA)_{40}$ tails at the 3′ ends and denoted by S-c-myb and MS-c-myb, respectively. All ODNs were synthesized at Hokkaido System Science (Hokkaido, Japan) and purified by high-pressure liquid chromatography. The fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco/BRL. Dulbecco's modified Eagle's medium (DMEM®) and RPMI1640 were obtained from Nissui Pharmaceutical Co., Ltd. Bovine serum albumin (BSA) was obtained from Sigma. The cell lines Colo205, LS174T, HepG2, and A375 were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Introducing Cholesterol. It is essentially important to introduce cholesterol only to the schizophyllan side chain. This is because the β-(1→3)-D-glucan main chain is necessary to form the complex with polynucleotides;[21] thus, it is better to keep the main chain intact during the reaction. We have already established such a fine chemical technique;[24] and this method was applied to introduce cholesterol, as presented in Fig. 2.

The 1,2-diol group in the s-SPG side chain was position-selectively cleaved and oxidized by sodium periodate ($NaIO_4$) in cold water at 4 °C. The conversion ratio to formyl groups was controlled by the concentration of $NaIO_4$. After being freeze-dried, followed by dialysis (fractionated molecular weight1/4 12000; Viskase Companies), partially formylated s-SPG was obtained. To introduce cholesterol, first we dissolved (the) partially formylated s-SPG, and 3β-cholest-5-en-3-yl *N*-(2-aminoethyl)carbamate (0.3 g) was also dissolved in DMSO, separately. The formylated SPG/DMSO was carefully added into the carbamate solution. The resultant mixture was stirred under nitrogen at room temperature. After 1 day, an excess amount of $NaBH_4$ (0.3 g) was added, and the mixture was stirred for another 2 days. After quenching un-reacted $NaBH_4$ with acetic acid, DMSO, and low-molecular compounds were removed by dialysis. After lyophilization of the resulting solution, the product was still crude, containing some unreacted cholesterol. The thus-obtained white solid was dissolved in DMSO, and the solution was slowly added into chloro-

form (the solution became turbid). The precipitated solid was corrected by filtration. This reprecipitation process was repeated three times. Finally, the precipitant was dissolved in DMSO, and then dialyzed with distilled water, repeatedly. Lyophilization of the solution gave cholesterol-appended SPG with satisfactory purity, confirmed by NMR. The percentage of cholesterol introduced was controlled with the oxidation level of SPG, and the modification level was determined by fluorescence-intensity titration using dansyl-D-leucine modified cyclodextrin[33] (see: the Supporting Information). These results and sample codes used in this paper are listed in Table 1.

Complexation between the Cholesterol-Appended SPG and AS ODNs. 2 mg of S-c-myb, MS-c-myb, or AS-c-myb was dissolved in 1 mL of 10 mM Tris buffer solution (pH = 7.8). An appropriate concentration of s-SPG/DMSO solution was added to the ODN solution, so that the water volume fraction was always adjusted to be 0.9 after mixing. The molar ratio ($M_{\text{s-SPG}}/M_{\text{ODN}}$) was controlled to be 1.5, where $M_{\text{s-SPG}}$ and M_{ODN} are the molar concentrations for the s-SPG and ODNs repeating units, respectively. After the mixture was kept at 5 °C for 1 night to complete complexation, DMSO was removed by ultrafiltration. After filtration, the final concentration of ODN was determined by measuring the ultraviolet absorbance. To confirm the complexation between ODN and s-SPG, we measured the circular dichromic (CD) spectrum, ultraviolet (UV) spectrum, and gel electrophoresis migration. The details for these experiments are described in a preceding paper in the series of this work.[13]

Antisense Assay; Measurement of Cell Proliferation. Cells were seeded in 96-well plates (Nunc) at a density of 2×10^4 cells/mL (1 well/100 μL) and allowed to attach to the plate for overnight. On the following day, the medium (MEM® supplemented with 0.1 mM nonessential amino acids and 10% FBS) was changed for a fresh medium, and the cells were treated with 25 or 50 μg/mL ODN, ODN/s-SPG complex (containing the same amount of ODN). Subsequently, the cells were incubated for 3 days before measuring of the cell growth. The cell number was evaluated by the use of Cell Counting Kit-8® (Dojindo, Japan), called the WST-8 assay.

Confocal Fluorescent Microscopy. A375 cells (9×10^3 cells) were grown just before confluence in a glass-bottom dish. Following the addition of a 1.09 mg/mL Texas Red®-labeled Chol(1)-SPG + 5′-FITC-labeled (dA_{50}) mixture (1 mL) to the medium (100 mL), the cells were incubated for 6 h at 37 °C in a 5% CO_2 incubator. The cells were then washed twice with PBS (125 mL), and fixed with 5% HCHO (90 mL) at 4 °C for 20 min. The cells were treated with one drop of the antifade solution and examined by fluorescence microscopy. Images of the samples were collected by fluorescence microscopy on IX-70 (Olympus, Tokyo, Japan), attaching a confocal scan unit CSU-10 (Yokogawa, Tokyo, Japan) with a ORCA-ER CCD Camera (Hamamatsu Photonics, Hamamatsu, Japan) using a 40 × Uapo/340 objective with NA 0.9. 5′-FITC-labeled (dA_{50}) phosphorothioate was obtained from Bex Ltd. Co., Tokyo, Japan, Texas Red® hydrazine and a Fluorescence antifade kit (SlowFade®) were purchased from Molecular Probes (Eugene, OR). The preparation of the Texas Red®-labeled SPG is described elsewhere.[5]

Reverse-Transcriptase-Mediated PCR. Reverse-transcriptase-mediated PCR (RT-PCR) was carried out to observe the sequence-specific silencing of mRNA for A375 cells. Primer sequences were as follows: c-myb, 5′-AAT TAA ATA CGG TCC CCT GAA 3′ (forward), 5′-TGC TCC TCC ATC TTT CCA CAG-3′ (reverse), 423 bp predicted product size; and β-actin, 5′-GGC TAC AGC TTC ACC ACC AC-3′ (forward), 5′-AGG GCA GTG ATC TCC TTC TG-3′ (reverse), 370 bp predicted product size. PCR products were resolved by agarose gel electrophoresis, and the band visualized by GelStar® was quantified by a gel documentation system (Alpha DigiDOC gel documentation system, Alpha Innotech). More details for the experiment are described elsewhere.[24]

Abbreviations. AS ODN; antisense oligodeoxynucleotide. AS-c-myb; 5′-GTGCCGGGGTCTTCGGGC-$(dA)_{40}$-3′ phosphorothioate, antisense ODN for c-myb mRNA. S-c-myb; 5′-CACGGCCCCAGAAGCCCG-$(dA)_{40}$-3′ phosphorothioate, sense ODN for c-myb mRNA. MS-c-myb; 5′-GT<u>C</u> C<u>T</u>G GGG TC<u>G</u> TCG GGC-$(dA)_{40}$-3′ phosphorothioate, a mismatched sequence for AS-c-myb, the mismatched bases are underlined. s-SPG; single chain of schizophyllan, without chemical modification. Chol(X)-SPG; cholesterol-appended schizophyllan, this work used four samples with different modification level, distinguished with X = 1, 2, 3, and 4 (Table 1). s-SPG/AS ODN or Chol(X)-SPG/AS ODN; complexes made from s-SPG and AS ODN, or Chol(X)-SPG and AS ODN. In this paper "/" means the complex. s-SPG + AS ODN; mixture of s-SPG and AS ODN. In this paper, s-SPG + AS ODN stands for a mixture of s-SPG + AS ODN; does not necessary mean the complex, it can be a just mixture of the two components without any interaction. After we confirmed the complex formation, we explicitly denote the complex as s-SPG/AS ODN.

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Supporting Information

Supporting information is available on line web; how the modification level of Chol(X)-SPG was estimated by titration with 6-(*N*-dansyl-D-leucylamino)-6-deoxy-β-cyclodextrin (Dansyl-leu-CD). This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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This paper is the 30th paper in the series of polysaccharide–polynucleotide complexes.

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