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Headline Articles

Enhancement of the Antisense Effect of Polysaccharide–Polynucleotide Complexes by Preventing the Antisense Oligonucleotide from Binding to Proteins in the Culture Medium#

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Schizophyllan is a 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-phosphodiester-polynucleotide is added to the schizophyllan/DMSO solution and, subsequently, DMSO is exchanged for water, the single chain of schizophyllan (s-SPG) forms a complex with the polynucleotide. In this paper, we report that phosphorothioate oligonucleotides can form a complex with s-SPG in the same manner as phosphodiester oligonucleotides. We carried out an in vitro antisense assay combining melanoma cell lines and a phosphorothioate antisense oligonucleotide (AS ODN) to depress *c-myb* mRNA. We found that the AS ODN bound to the complex reduces cell growth more efficiently than that of naked AS ODN by preventing the AS ODN from binding to albumin in the culture medium and being hydrolyzed.

Antisense oligonucleotides (AS ODNs) have been studied as a noteworthy strategy to suppress a particular gene expression.[1–3] Principles of antisense therapy are (1) to deliver AS ODN to the target cell, (2) to induce endocytosis of AS ODN, (3) to bind AS ODN to a particular mRNA to make a DNA/RNA duplex, and (4) to make RNase H cleave the duplex so as to inhibit protein expression. The major advantage of this strategy over conventional drugs is in the potential specificity of the action. Theoretically, an oligonucleotide can be designed to target any single gene in the human genome. Therefore, inhibition at the genetic level should be a more efficient intervention in the disease process than inhibition at the protein level. However, there are two major issues to overcome. The instability of AS ODNs in serum and the low up-take efficiency into the target cells.[3] The instability of AS ODNs is mainly ascribed to two factors: the hydrolysis mediated by deoxyribonuclease and non-specific binding to the serum proteins. The hydrolysis can be overcome by use of oligonucleotide analogues such as phosphorothioates, phosphoramidates, and peptide nucleic acids.[4] In particular, phosphorothioates are the leading candidate among the first generation of antisense compounds. Several of them are currently in phase I/II clinical trials.[5,6] Fortunately, phosphorothioates have an unexpected ability regarding cellular up-take, and currently, it is believed that cells acquire them though a receptor mediated endocytosis.[4,7]

Non-specific binding to proteins is the major obstruction in the case of delivering phosphorothioate AS ODN to the target cell. Several materials that can form a complex with phosphorothioates have been studied as AS ODN carriers.[3] Cationic lipids can form a complex with AS ODNs and encapsulate them in the liposome.[3,8] The liposomal delivery is one of the preferred methods, however, there are some drawbacks in its use. For instance, cationic liposomes tend to accumulate in the reticuloendothelial system, leading to a short life time in the serum. Furthermore, the size of the liposome is usually too large for cells.[3,8] Synthetic polycations, such as poly(L-lysine) and polyethyleneimine (PEI) have been studied as an AS ODN carrier, because polycations can form polyion complexes with polynucleotides.[3] Although polycations have a great advantage in improving transfection,[3,8] serious drawbacks have been pointed

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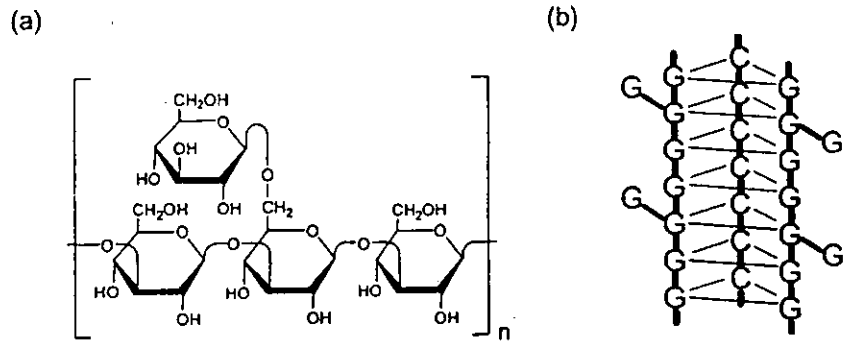

Fig. 1. Repeating units of schizophyllan (a), and the proposed interaction scheme based on our stoichiometry (b).[16] G and C denote the glucose and cytosine in the chains, respectively.

out, such as toxicity of the polycations and poor solubility of the resultant polyion complexes.[3,8] Graft-copolymers or block-copolymers consisting of polyethylene glycol and polycations have been proposed to improve the solubility.[9–12] However, cytotoxicity still remains a serious problem. Utilizing natural polysaccharides is considered to be a good and new approach to design an AS ODN carrier.[12] This is because natural polysaccharides can biodegrade into nontoxic components, provide solubility,[13] and be used as a cell-specific ligand.[14] Polysaccharides themselves can be used as an AS ODN binding moiety, because some glucans such as schizophyllan can form a complex with polynucleotides.[15,16]

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 every three glucose residues (see Fig. 1(a) for the chemical structure).[17] Schizophyllan adopts a triple helical conformation in water and a random coil in dimethyl sulfoxide (DMSO).[18–20] When water is added to the DMSO solution (renaturation), the triple helical structure can be partially retrieved, although the entire chain structure is not the same as that of the original triple helix.[21]

Recently, we found that the single chain of schizophyllan (s-SPG) forms a macromolecular complex with some homophosphodiester-polynucleotide [such as poly(C), poly(A), poly(U), poly(dA), and poly(dT)] when the polynucleotide is present in the renaturation process.[15,16,22] Our data showed that hydrogen bonds are formed between the glucose and the base moieties. Furthermore, other β-(1→3)-D-glucans, such as curdlan and lentinan, can form this same complex. Therefore, this complexation is characteristic for β-(1→3)-D-glucans.[23,24] Some other novel features for the complex include (1) the complex is remarkably stable (large binding constant) and considerably water-soluble in physiological conditions,[16] (2) the complexation occurs in a highly stoichiometric manner, and the stoichiometric number indicates that two schizophyllan units and three base units interact with each other [see Fig. 1(b)],[16] (3) when the s-SPG/DNA complex meets the corresponding complementary sequence, for example s-SPG/poly(dT) meets poly(dA), the complex dissociates immediately and hybridization takes place.[25]

When schizophyllan is used as an AS ODN carrier, the three features of (1)–(3) mentioned above are advantageous. This paper presents experimental evidence that the AS ODN in the complex executes its task more efficiently than that of naked AS ODNs, using cutaneous melanoma cell lines and a phosphorothioate AS ODN to target the disruption of *c-myb* protooncogene.[26] *c-myb* is overexpressed in many melanoma cell lines and plays a central role in the exponential growth of this malignant neoplasm.[27,28] Depression of *c-myb* can be done using an AS ODN addressed to a targeted region of *c-myb* mRNA, the most effective antisense sequence of which is well-known.[28,29] Therefore, the *c-myb* antisense has been a good model assay system in antisense research.[29–31]

Results and Discussion

Complexation between Phosphorothioates and Schizophyllan. Our previous studies[15,16,23,32] have shown that once s-SPG combines with phosphodiester oligonucleotides, the circular dichromic (CD) spectrum is drastically changed and a hypochromic effect is observed in the UV spectrum. These changes occur in the 200–400 nm wavelength range. Schizophyllan has no functional group to absorb the light in this range. On the other hand, the base moieties in nucleotides do have an absorbance. Therefore, the changes in CD and UV can be related to the conformational transition in polynucleotides, hence they can be used as a criterion for the complex formation. Figure 2(a) compares the CD spectrum of naked phosphorothioate AS-c-myb with that of a mixture of AS-c-myb and s-SPG (coded by AS-c-myb + s-SPG[33]) measured at 5 °C. The spectrum for AS-c-myb + s-SPG has two positive bands at 260 and 280 nm, while AS-c-myb has a broad band centered around 270 nm, indicating that the AS-c-myb/s-SPG complex is formed. Figure 2(b) presents the temperature dependence of the UV absorbance at $\lambda = 260$ nm for AS-c-myb itself and AS-c-myb + s-SPG at the same nucleotide molar concentration (20.2 μM, 6.67 μg/mL). At 5 °C, the absorbance of the mixture is lower than that of naked AS-c-myb because of hypochromism due to the complexation. With increasing temperature, the absorbance of naked AS-c-myb increases due to a decrease in the number of stacked bases. On the other hand, the absorbance for the mixture stays at 1.88 in the temperature range of 5–40 °C, then starts to increase with further increases in temperature above 50 °C, and finally merges into that of naked AS-c-myb above 75 °C. This behavior can be explained as follows. (1) In the range of 5 °C < T < 40 °C, the constant absorbance means that the complex conformation does not change upon heating, (2) at 40 °C, the complex starts to dissociate, therefore the absorbance increases. (3) T > 75 °C, the complex dissociates completely, thus the absorbance of the mixture is identical

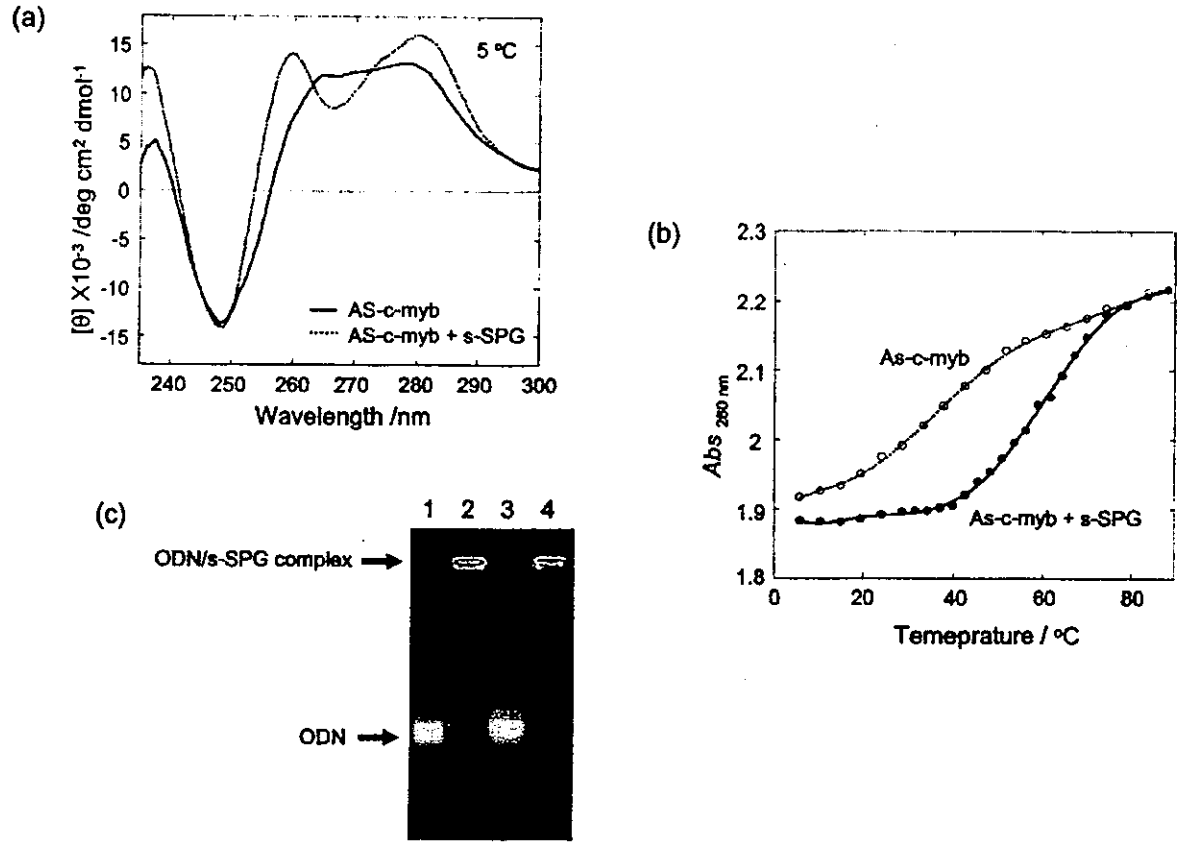

Fig. 2. Confirmation of the complex formation between AS-c-myb and s-SPG complex: (a) The CD spectra of AS-c-myb and its mixture with s-SPG, (b) the temperature dependence of the UV absorbance, (c) comparison of the gel migration patterns between AS-c-myb and its mixture with s-SPG. In the panel (c), 2 wt % NuSieve agarose gel (BMA) was used and the gel was stained with GelStar® (BMA). Lane 1: AS-c-myb, lane 2: AS-c-myb + s-SPG, lane 3: S-c-myb, and lane 4: S-c-myb + s-SPG.

to that of naked AS-c-myb. The results presented in Fig. 2(b) indicate that the complex is stable at both room and cell-incubation (37 °C) temperatures.

Figure 2(c) compares the gel electrophoresis migration patterns between naked AS-c-myb and AS-c-myb + s-SPG (or naked S-c-myb and S-c-myb + s-SPG). Here, only the ODNs are stained by GelStar®. The ODNs themselves migrate, however, the mixture of the ODNs and s-SPG do not at all. This feature also provides evidence of complex formation in the mixture.[16,32]

Proliferation after Administrating AS OND and s-SPG. Figure 3 shows the relation between the number of cells (estimated by WST-8 assay) and the culture days after the melanoma A375 cells were treated with AS-c-myb, AS-c-myb/s-SPG, s-SPG, or saline (control). Here, the dose weight ratio of s-SPG:AS ODN was always fixed at 150 μg:50 μg, and the total doses for AS ODN, s-SPG, and AS ODN/s-SPG were 50, 150, and 200 μg/mL, respectively (i.e., the amount of the AS ODN dose was always kept at 50 μg). The number of the cells increased drastically (probably exponentially) when the cells were exposed to s-SPG and the saline reference, and the cell number was identical between s-SPG and the reference. This means that s-SPG itself does not have any cytostatic effect at this dose. When the cells were treated with AS-c-myb and AS-c-myb/s-SPG, the proliferation for both systems was reduced compared with the control. After 5 days, the number

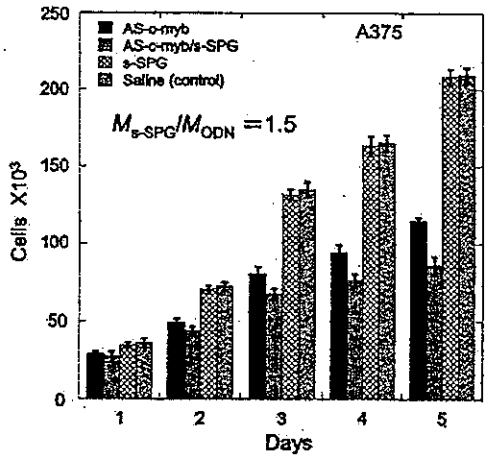

Fig. 3. Comparison of the cell growth after treated melanoma A375 cells with AS-c-myb, AS-c-myb/s-SPG, and s-SPG, comparing with the control assay in which only saline was added. The cells were grown in the presence of 50 μg/mL of AS-c-myb, 200 μg/mL of AS-c-myb/s-SPG (containing 50 μg/mL AS-c-myb), and 150 μg/mL of s-SPG at 37 °C. Cell number was determined by WST-8 assay. The molar concentration ratio ($M_{s\text{-}SPG}/M_{poly(dA)}$) is fixed at 1.5.

of cells for these systems becomes almost half that of the control. Furthermore, AS-c-myb/s-SPG shows a lower proliferation than naked AS-c-myb itself at any time, suggesting that the antisense activity is more enhanced for the complex than for the naked ODN. From this experiment, three culture days are long enough to provide the distinguishable difference in the proliferation. Thus, hereinafter we will compare the proliferation only after three days and, for convenience, the number of cells is normalized by that of the control, and the resultant value was defined as the cell growth.

Figure 4 shows the relationship between cell growth and the s-SPG:AS-c-myb molar ratio. Here, the AS-c-myb dose is fixed at 50 μg/mL and only the s-SPG dose is changed from 0 to 1000 μg (corresponding to a molar ratio of 10, where the repeating unit molecular weights of AS-c-myb and s-SPG are 330 and 650, respectively). The cell growth decreases with an increasing ratio and reaches a minimum around the ratio = 1–2, then abruptly increases. As mentioned in the introduction, the complex consists of two s-SPG repeating units and three base units (see Fig. 1(b)), thus the stoichiometric ratio is 2/3. The molar ratio to give the minimum is larger than the stoichiometric ratio. Hereinafter we will evaluate the cell growth at the molar ratio = 1.5.

When the molar ratio was at 5 or 10, the AS-c-myb/s-SPG solution was found to be slightly opaque owing to precipitation of excess s-SPG. Therefore, the increase in the cell growth at the larger ratios can be explained as such that some AS-c-myb molecules co-precipitated with s-SPG, thus the effective AS-c-myb concentration in the solution decreased.

Figure 5 presents the s-SPG dose dependence of the cell growth for C32 and A375 cells (without AS ODN) over the s-SPG dose range from 18.75 to 600 μg/mL. The cell growth decreases slightly, by 10–7% at 600 μg/mL, indicating that there is a slight cytostatic-effect for s-SPG when excess is added. When we examined the antisense effect in this work, most experiments were carried out in an s-SPG dose range of less than 300 μg/mL, an amount of s-SPG that reduces cell growth by no more than 5%. On the other hand, the AS ODN reduces the cell growth by about 50% (see Fig. 4). Therefore, the cytotoxicity of s-SPG is negligible when we discuss the antisense effect.

Dose Dependence of Proliferation. Figure 6 shows the

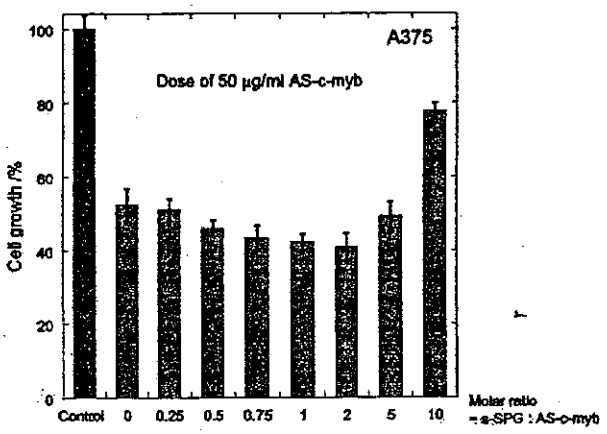

Fig. 4. Relation between the cell growth and s-SPG:AS-c-myb molar ratio for A375. The AS-c-myb dose was fixed at 50 μg/mL.

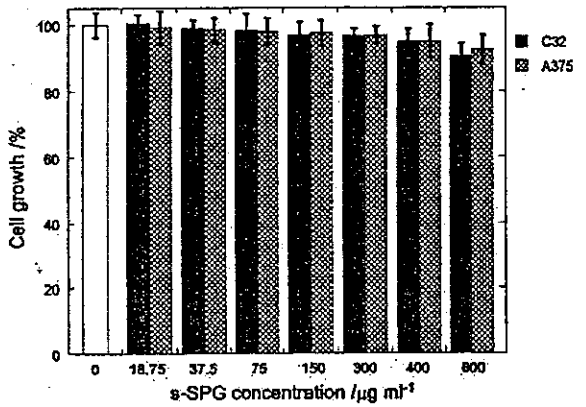

Fig. 5. Cellular toxicity for schizophyllan dose for C32 and A375 cells.

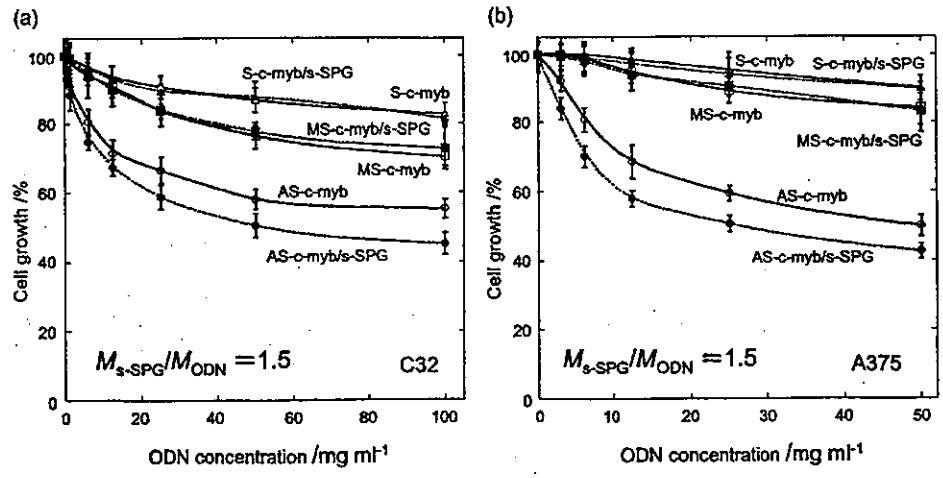

Fig. 6. Dose dependence of the melanoma cell growth for C32 (a) and A375 (b), when treated with AS-c-myb (○), S-c-myb (△), MS-c-myb (□), AS-c-myb/s-SPG (●), S-c-myb/s-SPG (▲), and MS-c-myb/s-SPG (■). The cell number was determined after 3 days. The molar concentration ratio ($M_{s\text{-}SPG}/M_{poly(dA)}$) is fixed at 1.5.

Table 1. Statistical Analysis of the Antisense Test for the Cell Growth at 100 mg/mL Dose for C32

	S-c-myb	S-c-myb/s-SPG	MS-c-myb	MS-c-myb/s-SPG	AS-c-myb	AS-c-myb/s-SPG
Average	82.7	83.0	73.0	72.6	56.4	45.0
Dispersion	10.9	16.6	10.0	28.7	4.3	8.5
n	4	4	5	5	5	5
T-value	0.47		0.21		6.5	

ODN dose dependence of the cell growth for the C32 (Panel a) and A375 (Panel b) cells. Here, the ODN dose was controlled by changing the ODN concentration added to the plate. To confirm the antisense effect, the sense sequence (S-c-myb), its complex (S-c-myb/s-SPG), the mismatch sequence (MS-c-myb), and its complex (MS-c-myb/s-SPG) were examined as well as AS-c-myb and AS-c-myb/s-SPG. For each 100 μg/mL dose, we calculated the dispersion and T-value, summarized in Table 1. As shown by the figure, the general trends for both cell lines are consistent with each other. AS-c-myb and AS-c-myb/s-SPG reduce the cell growth with increasing ODN concentration and growth decreases 45–50% at the highest dose for both lines. Although both (AS-c-myb and AS-c-myb/s-SPG) reduce the growth rate drastically, it is clear that AS-c-myb/s-SPG reduces the cell growth more efficiently than naked AS-c-myb. As presented in Table 1, the T-value between AS-c-myb and AS-c-myb/s-SPG is much larger than the others, indicating that the complex with AS-c-myb causes a statistically significant difference. On the other hand, S-c-myb and S-c-myb/s-SPG show a relatively small reduction and the growth decreases to only 85–90% at the highest dose. Furthermore, the growth between S-c-myb and S-c-myb/s-SPG is similar at the same ODN concentration, confirming the negligibly small cytotoxicity for s-SPG. Even the sense sequence reduces the growth. According to the previous work, phosphorothioate DNAs tend to depress cells growth in a nonspecific manner.[4,34–37] Therefore, this simple cytotoxicity effect should be the main reason for the decrease in cell growth for S-c-myb and S-c-myb/s-SPG. When we exposed both cells to the G-rich mismatch sequence (MS-c-myb and MS-c-myb/s-SPG), the growths were lowered more than those corresponding to the sense sequence (i.e, S-c-myb and S-c-myb/s-SPG). This can be explained by anti-proliferation activity for the contiguous G residues.[38] Figure 6 demonstrates that administering AS-c-myb decreases proliferation, and this effect is beyond both the cytotoxicity of the phosphorothioates and the non-antisense effect of the G-rich sequence. The most important result in this experiment is that the complex always provides a more enhanced anti-proliferation effect than naked AS ODN.

Although the data are not shown, when we carried out the same experiment as shown in Fig. 6(b) using the phosphodiester AS-c-myb (instead of phosphorothioate), the antisense effect was not as clearly observed in comparison with the phosphorothioate system. The poor antisense effect was not improved much even when we used the corresponding complex. The growth was 70–80% at a 50 μg/mL dose for the A375 cells. These features may be ascribed to the fact that phosphorothioate is taken up by cells much easier than phosphodiester, because the surface protein responsible for ODN up-take has a higher affinity for phosphorothioate than for phosphodiester.[4,7,39,40]

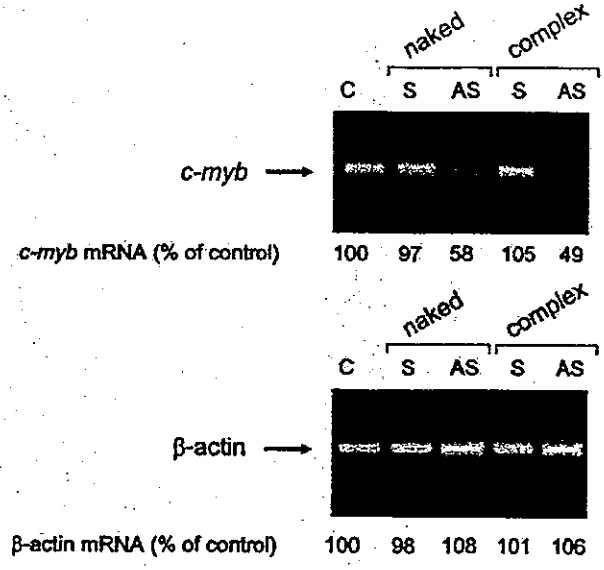

Fig. 7. Expression of β-actin and *c-myb* mRNA determined by RT-PCR analyses. A375 cells were cultured in 24-well plates at initial concentration of 2×10^4 cells/mL in medium, and were treated with naked ODNs (60 μg/mL) and ODNs/s-SPG (containing 60 μg/mL AS-c-myb) for 2 days before extraction of RNA. Extracted RNA was subjected to RT-PCR and amplified DNA fragments were run on 1.5% (w/v) agarose gels. Lane C: untreated cells (control), lane S: S-c-myb or S-c-myb/s-SPG, lane AS: AS-c-myb or AS-c-myb/s-SPG. Amplified DNA fragments were quantified by Alpha DigiDOC gel documentation system.

Confirmation of the Antisense Effect by Reverse-Transcriptase-Mediated PCR. The enhanced anti-proliferation effect for AS-c-myb/s-SPG in Fig. 6 strongly supports that the antisense ODN depresses *c-myb* expression. Encouraged by these results, we examined whether naked AS-c-myb and its complex carried out their task well in eliminating the target mRNA in a sequence-specific manner using RT-PCR. The results are presented in Fig. 7. First of all, we evaluated the amount of β-actin mRNA, comparing the control (no administration of ODN) with the administration of S-c-myb, S-c-myb/s-SPG, AS-c-myb, and AS-c-myb/s-SPG. As shown in the lower panel in Fig. 7, there is no difference among the samples, indicating that the administration of these ODNs does not have any effect on the amount of β-actin mRNA. However, when we isolated the *c-myb* mRNA and evaluated the amount of mRNA by the same manner (the upper panel in the figure), the AS-c-myb and AS-c-myb/s-SPG did decrease the amount of mRNA, while both AS-c-myb and AS-c-myb/s-SPG did not. This result indicates that antisense ODN eliminated the target mRNA in a sequence-specific manner. Moreover, the com-

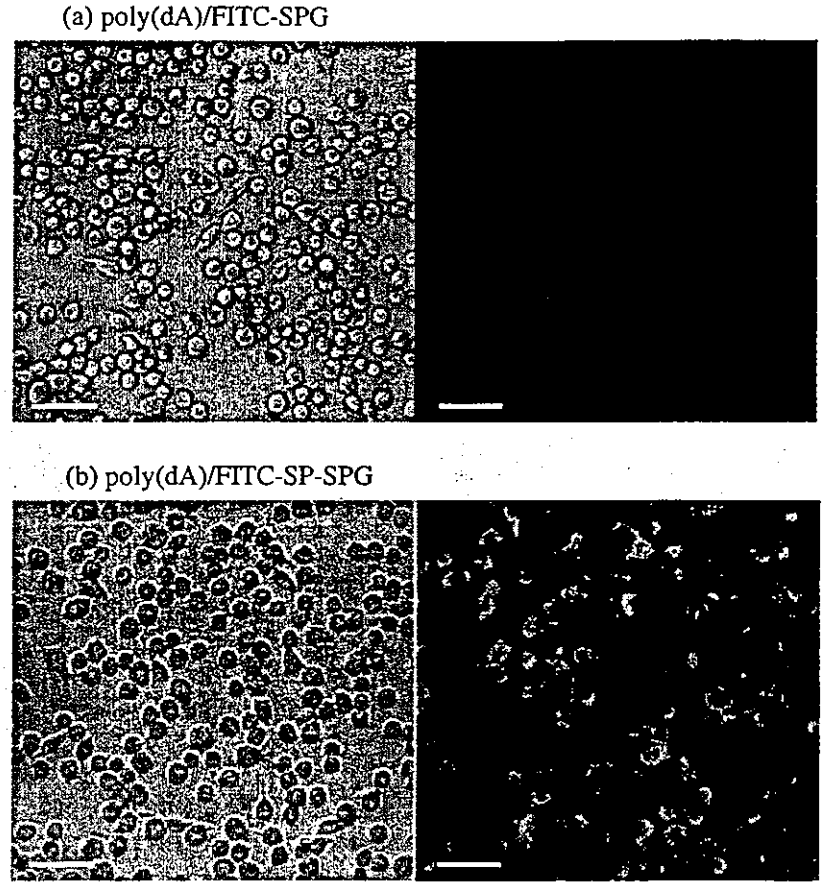

Fig. 8. Confocal fluorescent microscopy images when A375 cells were treated with poly(dA)/FITC-SPG complex (a) and poly(dA)/FITC-SP-SPG (b). Phase contrast images of the same field of cells are shown left each fluorescence image. The bar indicates 100 μm.

plex reduces the amount more drastically than the naked one, confirming the antisense effect is enhanced in the complex.

Up-Take of s-SPG and ODN. One of the possible ways to explain the enhanced antisense effect of the complex is that schizophyllan may increase the up-take efficiency. To examine whether s-SPG is taken into a cell, we exposed A375 cells to a complex made from poly(dA) and FITC-SPG (fluorescein isothiocyanate-labeled schizophyllan) and observed the cell morphology with confocal fluorescent microscopy. Figure 8(a) shows a typical image obtained in this experiment. For comparison, we exposed the same cells to a complex made from poly(dA) and FITC-SP-SPG[41] (FITC-labeled, and spermine-appended schizophyllan) which was already observed to have an increase in the cellular uptake due to the positive charge, as seen in the confocal microscopic image in Fig. 8(b). Figure 8(b) clearly shows that the labeled materials are taken into a cytosol. On the other hand, there is no such a feature observed in Fig. 8(a). Therefore, we conclude that schizophyllan itself has no capability to induce endocytosis.

Nuclease-Mediated Hydrolysis of the Antisense ODNs. Figure 9 plots ΔAbs against the incubation time during the hydrolysis by nuclease P_1 for AS-c-myb [AS-c-myb] and the complex made of s-SPG and AS-c-myb [AS-c-myb/s-SPG]. The nuclease P_1 effectively hydrolyzes single stranded DNAs and produces 5′-monophosphoric nucleic acids, resulting in increasing ΔAbs.[42,43] Figure 9 shows that the complexation decreases the ΔAbs change more effectively than the naked samples, confirming our previous conclusion that the complex can protect the bound DNA form a nuclease attack.[44]

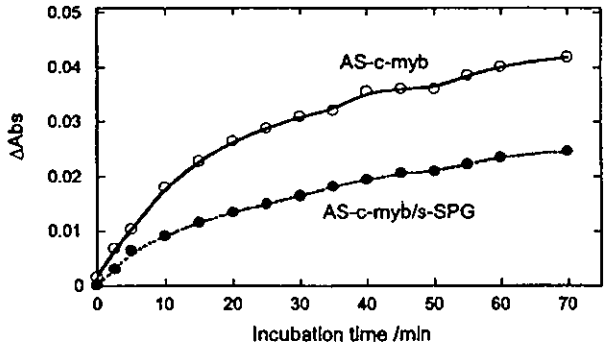

Fig. 9. Increment of the absorbance during the hydrolysis by nuclease P_1 at 37 °C, comparing phosphodiester AS-c-myb [AS-c-myb (PO)], phosphorothioate AS-c-myb [AS-c-myb], and the complex made of s-SPG and phosphorothioate AS-c-myb [AS-c-myb/s-SPG]. 15.2 μM of AS-c-myb was added to 2 mL of 3 mM sodium acetate (pH 6.0), and 1 mM $ZnSO_4$. The concentration of nuclease P_1 was 40 U/mL.

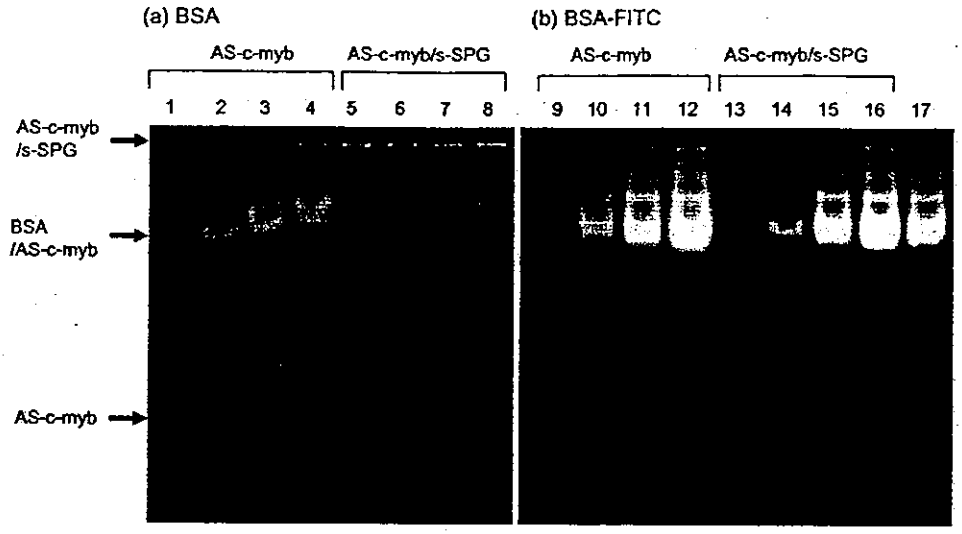

Fig. 10. AS-c-myb/s-SPG complex stability in the presence of BSA determined by gel shift assay. (a) BSA was mixed with AS-c-myb or AS-c-myb/s-SPG complexes. These mixtures were incubated for 30 min at 37 °C, and run on a 7.5% non-denaturing polyacrylamide gel. The gel is stained by GelStar®, and the image was obtained by a gel documentation system (Alpha DigiDOC gel documentation system, Alpha Innotech). Lane 1–4: naked AS-c-myb (0.2 μg), lane 5–8: AS-c-myb/s-SPG (containing 0.2 μg AS-c-myb). Lanes 2 and 6: added 10 μg BSA, lanes 3 and 7: 50 μg BSA, and lanes 4 and 8: 100 μg BSA. (b) BSA-FITC was mixed with AS-c-myb (9–12) or AS-c-myb/s-SPG complexes (13–16). The gel is not stained by GelStar(r). Lane 9: AS-c-myb itself (0.2 μg), no BSA-FITC, lane 13: AS-c-myb/s-SPG (containing 0.2 μg AS-c-myb), no BSA-FITC, lanes 10 and 14: added 10 μg BSA-FITC, lanes 11 and 15: 50 μg BSA-FITC, and lanes 12 and 16: 100 μg BSA, lane 17: 50 μg BSA-FITC itself.

Stability of the Complex in the Cell-Culture Medium. Figure 10(a) compares the gel-shift between naked AS-c-myb and AS-c-myb/s-SPG in the presence of BSA. In the panel (a), the gel was stained with GelStar®, thus only nucleonic bases can be observed. In the absence of BSA, the naked AS-c-myb (0.2 μg) migrates a great distanse (lane 1). When we added 10 μg of BSA to 0.2 μg of AS-c-myb (lane 2, BSA concentration is about 1 mg/mL), there was still a small amount of the free AS-c-myb. However, most of them were incorporated into the BSA/AS-c-myb complex. When we added extra BSA (50 and 100 μg in lanes 3 and 4, respectively, i.e., BSA concentrations were about 5 and 10 mg/mL), there was no free AS-c-myb, and all ODN were bound to BSA. When we carried out a similar experiment for the AS-c-myb/s-SPG complex (0.2 μg of AS-c-myb and 0.6 μg of s-SPG), the addition of BSA provided essentially no change in migration. Lane 5 shows the complex itself (i.e., without BSA), stays at the starting hole.[16,32] When we added 10 μg of BSA to 0.2 μg of AS-c-myb (lane 6), the migration pattern was exactly the same as that of the complex itself. When we increased the BSA amount to excess, some AS-c-myb seemed to be taken out of the complex and bound to BSA, however, this amount is relatively small compared with that in the complex. Although the data are not shown, after we mixed the complex with serum and waited a few days, we obtained the same results as those in Fig. 10(a).

We carried out the same experiments as those in Fig. 10(a) using fluorescein-labeled BSA (BSA-FITC), where only BSA was observed. Figure 10(b) shows the results of the comparison between naked AS-c-myb (lanes 9–12) and the AS-c-myb/s-SPG complex (lanes 13–16). BSA-FITC was added at 0, 10, 50, and 100 μg for lanes 9 and 13, 10 and 14, 11 and 15, and 12 and 16, respectively. In lane 17, only BSA-FITC (50 μg) was loaded. As shown in the figure, there is no difference in the migration pattern between the naked ODN and the complex. This feature indicates that the complex is not bound to BSA, otherwise the BSA would be in the staring hole.

Based on the above results, we conclude that AS-c-myb/s-SPG is considerably stable in a solution containing an extra amount of BSA, and that the complex remains intact in the presence of BSA. On the other hand, naked AS-c-myb is bound to BSA. The BSA concentration range examined here covers that for the cell-culture medium. Therefore, the complexed AS-c-myb hardly bound to BSA when we cultured the cells.

A Possible Mechanism for the Enhanced Antisense Effect for the Complex. Our culture medium contains an albumin, BSA, a necessary ingredient for growth of the cells, as well as some nuclease. It is well-known that BSA itself easily binds to hydrophobic molecules. In particular, phosphorothioate is more hydrophobic than phosphorodiesters,[45–47] so BSA should bind to phosphorothioate strongly. In fact, Fig. 10(a) shows the strong affinity between the naked ODN and BSA. For human leukemia cells, there is evidence that AS ODN is up-taken by receptor-mediated endocytosis relating to the albumin recognition site on the cell surface.[45] If the albumin mediated endocytosis was the major route for the up-take of AS-c-myb in our system, it is difficult to explain the enhanced antisense effect of the complex, because the AS-c-myb in the complex can not bind to BSA.

It is surprising that the cellular up-take of phosphorothioate ODNs occurs to a greater extent than would be expected on the basis of charge and size consideration. In fact, the phosphodiester AS ODN shows a poor antisense effect in our system. The pharmacokinetics of phosphorothioate ODNs, especially its cellular up-take mechanism, are not well understood. However, it has been suggested that the up-take of phosphorothioate AS ODNs can be mediated by pinocytosis or adsorptive endo-

cytosis facilitated by proteoglycans and the binding proteins in the cell membrane.[4,39,40] Therefore, it seems that the presence of AS ODN free from BSA is important, and since the complex can prevent the AS ODN from binding to BSA, the antisense effect was enhanced in our system. Furthermore, the complex can protect the bound AS ODN from nuclease attack. In summary, we can conclude that the antisense effect is enhanced in the complex by protecting AS ODN from unfavorable interactions (such as hydrolysis or absorption) with proteins in the culture medium.

Conclusion

The present report describes the formation of a phosphorothioate–oligonucleotide complex with s-SPG in the same manner as phosphodiester oligonucleotides. Furthermore, we carried out an in vitro antisense assay combining melanoma cell lines and a phosphorothioate antisense oligonucleotide (AS ODN) to depress *c-myb* mRNA, and found that the AS ODN bound in the complex reduces cell growth more efficiently than that of naked AS ODN. The reason for the enhanced depression has been rationalized by a new proposal that states the complex can prevent the AS ODN from binding to BSA in the culture medium as well as protecting AS ODN from nuclease. The present results show, therefore, that schizophyllan can be used as an AS ODN carrier.

Experimental

Materials. 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.[16,17] The oligonucleotide (ODN) sequence of 5′-GTG CCG GGG TCT TCG GGC-3′ is well known to bind to *c-myb* mRNA and lead to the depression of *c-myb*.[26,28–31] However, since short and hetero ONDs cannot bind with s-SPG,[16,32] we had to attach a poly(dA) tail with 40 bases at the 3′ end of this sequence. Thus, in this study, 5′-GTG CCG GGG TCT TCG GGC-$(dA)_{40}$-3′ phosphorothioate was used as an AS ODN and is denoted by AS-c-myb, hereafter. To examine whether the antisense effect really strives to suppress the cell growth, we used an ODN containing the sense sequence 5′-GCC CGA AGA CCC CGG CAC-3′[28,29] as a reference. Additionally, the antisense phosphorothioate ODN can show anti-proliferation activity through a non-antisense path when the sequence contains some contiguous G residues.[38] Therefore, as a second control, a mismatch bases of AS-c-myb; 5′-GTC CTG GGG TCG TCG GGC-3′[30] was used (the mismatched sequences are underlined). For the same reason as those in the antisense sequence, the sense and mismatched sequences were connected to the $(dA)_{40}$ tails at the 3′ ends and denoted by S-c-myb and MS-c-myb, respectively. AS-c-myb, S-c-myb, and MS-c-myb were synthesized at Hokkaido System Science (Hokkaido, Japan) and purified with high-pressure liquid chromatography.

MEM® (minimum essential medium), nonessential amino acids, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco/BRL. Dulbecco's modified Eagle's medium (DMEM®) was obtained from Nissui Pharmaceutical Co. Ltd. Bovine serum albumin (BSA) was obtained from Sigma. Fluorescein isothiocyanate (FITC) was purchased from Dojin, Japan. An ultrafiltration device (Centricon® plus-10; NMWL, 10000) was purchased from Millipore (Bedford, MA). Poly(dA) was purchased from Amasham (Kyoto, Japan).

Fluorescein-appended BSA (BSA-FITC) was synthesized by mixing a BSA (109 mg, 1.7 μmol)/water solution with a fluorescein-4-isothiocyanate (0.7 mg, 1.8 μmol)/acetone solution (Doujin, Japan). The mixture was purified by dialysis with distilled water several times and then lyophilized. Spermine-appended SPG (SP-SPG) was synthesized as described in our previous paper.[41] FITC-labeled schizophyllan (FITC-SPG) and FITC-labeled SP-SPG (FITC-SP-SPG) were prepared as follows. Schizophyllan (60 mg) was dissolved in purified DMSO (2 mL). To this solution was added FITC (1 mg), and the mixture was vigorously stirred in the dark at room temperature for 60 h. The resulting derivative was purified by ultrafiltration with purified DMSO (3 mL) to remove excess FITC. The solutions were lyophilized to obtain FITC-SPG and FITC-SP-SPG.

Preparation of Complex between ODN and s-SPG. 2 mg of S-c-myb, MS-c-myb, and AS-c-myb were 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 0.9 after mixing. The molar ratio ($M_{s\text{-SPG}}/M_{ODN}$) was controlled at 1.5, except when we measured the $M_{s\text{-SPG}}/M_{ODN}$ dependence of the cell growth (Fig. 4), where $M_{s\text{-SPG}}$ and M_{ODN} are the molar concentrations for the s-SPG and ODNs repeating units, respectively. After the ODN and s-SPG mixture was kept at 5 °C for 1 night to lead the complexation, DMSO was removed by ultrafiltration. After the filtration, the final concentration of ODN was determined by measuring the ultraviolet absorbance. To confirm the complexation between ODNs and s-SPG, we measured the circular dichroism (CD) spectrum, ultraviolet (UV) spectrum, and gel electrophoresis migration pattern. The details for these experiments are described in the preceding paper in this series of work.[15,16,23–25,32]

Cell Culture and Confocal Fluorescent Microscopy. The melanoma cell lines C32 and A375 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The C32 cells were maintained in MEM® supplemented with 0.1 mM nonessential amino acids and 10% FBS. The A375 cells were maintained in DMEM® supplemented with 10% FBS. All medium contained a 1 wt % penicillin/streptomycin mixture. Cell incubation was always carried out at 37 °C in fully humidified air containing 5 wt % CO_2.

A375 cells (2×10^5 cells) were grown just before confluence in a 96-well tissue culture plate (NUNC). The FITC-SPG–poly(dA) complex and FITC-SP-SPG–poly(dA) complex [the molar ratio ($M_{s\text{-SPG}}/M_{poly(dA)}$) was controlled to be 1.5] were added to the medium (final concentration 200 μg/mL). The cells were incubated for 12 h at 37 °C in a 5% CO_2 incubator. The cells were then washed three times with PBS (100 μL) and examined by fluorescence microscopy. Images of the samples were collected by fluorescence microscopy on an ECLIPSE TE2000-U (Nikon, Tokyo, Japan) attached to a confocal scan unit Radiance 2100C (Bio-Rad, Tokyo, Japan) using a 20× objective lens (the total magnification was 200×).

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 overnight. The following day, the medium (MEM® supplemented with 0.1 mM nonessential amino acids and 10% FBS) was changed to fresh medium, and cells were treated with an appropriate amount of ODN (0.39–100 μg/mL), ODN/s-SPG complex (containing 0.39–100 μg/mL ODN), or s-SPG (1–200 μg/mL). Subsequently, cells were incubated for 1, 2, 3, 4, or 5 days before measurement of cell growth. The cell number was evaluated by use of a Cell Counting Kit-8® (Dojindo,

Japan) called the WST-8 assay. The WST-8 assay uses a novel tetrazolium salt 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8), which produces a water-soluble formazane dye upon reduction mediated by dehydrogenase in living cells.[47] After incubation for 1, 2, 3, 4, or 5 days, 10 μL of the Cell Counting Kit-8 working solution (containing WST-8 and 1-methoxy-5-methylphenazinium methosulfate) was added to each well, followed by incubation for 4 h at 37 °C. Plates were read on a microplate reader Multiskan JX (Thermo Labsystems) using a wavelength of 450 nm in comparison with 650 nm. Each set of control or treated cells was tested in triplicate wells, and the mean and standard deviation of the values was plotted. All growth studies were carried out at least twice.

Nuclease-Mediated Hydrolysis. We measured the increment in the ultraviolet absorbance (ΔAbs) at 260 nm during the hydrolysis of the nucleotides with a JASCO V-570. The hydrolysis of AS-c-myb by nuclease P_1 (from *Penicillium citrium*, Sigma)[42,43] was carried out at 37 °C in a reaction mixture in which 15.2 μM of AS-c-myb was added to 2 mL of 3 mM sodium acetate (pH 6.0) and 1 mM $ZnSO_4$. The concentration of nuclease P_1 was 40 U/mL.

Isolation of Total RNA and Reverse-Transcriptase-Mediated PCR. To confirm that the reduced proliferation was due to decreasing *c-myb* expression by the antisense effect, the expression of *c-myb* was evaluated at the mRNA level by the reverse-transcriptase-mediated PCR (RT-PCR) method.[48–50] A375 cells were plated in 24-well plates (NUNC) at an initial concentration of 2×10^4 cells/mL in the medium and allowed to attach to the plate overnight. The following day, the cells were treated with 60 μg/mL ODN and ODN/s-SPG complex. After 2 days, the cells were harvested with 0.25% trypsin-EDTA in PBS. The total RNA was isolated with a RNeasy Mini Kit (QIAGEN) as recommended by the manufacturer. RT-PCR was performed with a ThermoScript RT-PCR system (Invtrogen). A PCR tube contained RNA (0.9 μg), oligo (dT) primer, dNTPs (10 mM, 2 μL), DTT (0.1 M, 1 μL), and RNaseOUT (2 U/μL, Thermoscript RT (0.75 U/μL). Synthesis of the first-strand cDNA was done at 55 °C for 40 min in a DNA thermal cycler (T gradient, Biometra, Germany). The resulting cDNA fragments were amplified with 2 U/ml of *Taq* polymerase, $MgCl_2$ (50 mM, 1.6 μL), dNTPs (10 μM, 1 μL), and 15 pmol of each primer. Regents were from Invitrogen. Thermal cycling consisted of 1 min denaturing at 95 °C, 1 min annealing at 55 °C (*c-myb*) or 57 °C (*β*-actin), and 1 min of extension at 72 °C. This was repeated for 40 cycles (*c-myb*) and 28 cycles (*β*-actin), with a final extension period of 3 min. 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. *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. PCT 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).

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This paper is the 13th paper in the series of polysaccharide–polynucleotide complexes.

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33 In this paper, we use the code "AS-c-myb + s-SPG" to show a mixture of AS-c-myb and s-SPG. It does not always mean that the complex is formed in this mixture. When necessary, the complex is denoted by "AS-c-myb/s-SPG".

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A Polysaccharide Carrier for Immunostimulatory CpG DNAs To Enhance Cytokine Secretion[‖]

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Oligodeoxynucleotides containing unmethylated CpG sequences (CpG DNA) have been shown to stimulate a cell-mediated immune response for mammals.[1] This immune response is considered to be a defense system that mammals have evolved because unmethylated CpG sequence emerges more frequently in bacterial DNAs than in mammalian DNAs. Considerable attention is devoted to this response because CpG DNA can be extraordinarily effective adjuvants for many vaccines against infections agents, cancer antigens, and allergens.[1] Henmi et al.[2] demonstrated that CpG DNA can be recognized by Toll-like receptor 9 (TLR-9). The Toll-like receptor family is generally located on plasma membrane and can recognize bacterial cell wall components. However, Ahmad-Nejad et al.[3] demonstrated that TLR-9 is not localized on the cell surface, but intracellularily, predicting that if we can efficiently deliver CpG DNA to endosome and/or lysosome, the immune response can be enhanced and controlled artificially.

To deliver CpG DNA to intracellular endosome and/or lysosome, there are two major issues to overcome: instability of CpG DNA in biological fluids and low uptake efficiency into cells, similar issues for antisense DNA carriers.[5] Instability of CpG DNA is ascribed to hydrolysis mediated by deoxyribonuclease. The hydrolysis can be significantly suppressed by the use of phosphorothioates,[5] and thus many studies use phosphorothioate CpG DNAs. However, phosphorothioates can be bound to some plasma proteins in nonspecific manners, which can cause undesirable side effects. Therefore, CpG DNA carriers are required to prevent the phosphorothioates from forming such nonspecific interactions with proteins, as well as to protect them against hydrolysis mediated by deoxyribonuclease. Several materials have been studied as CpG DNA carriers. The liposomal delivery is one of the preferred methods;[6] however, there are some drawbacks in its use.[4]

Sakurai and Shinkai found that the β-(1 → 3)-D-glucan schizophyllan (SPG) forms a novel complex with some polynucleotides,[7] and the complex is applicable to an antisense DNA carrier.[8] Here, SPG is an extracellular polysaccharide produced by the fungus *Schizophyllan commune*, and the main chain consists of β-(1 → 3)-D-glucan and one β-(1 → 6)-D-glycosyl side chain linked to the main chain at every three glucose residues (Figure 1).[9] 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.[10] This pH response seems ideal for releasing the complexed CpG DNA in late endosome in which the pH is maintained about 5.0. The complexed oligonucleotides acquire stronger resistance to deoxyribonuclease-mediated hydrolysis and SPG can prevent the complexed phosphorothioate oligonucleotides from forming nonspecific interactions with plasma proteins.[11] These properties seem greatly advantageous as a CpG DNA carrier, and the main purpose of this communication is to present preliminary results to prove superiority of SPG for the carrier.

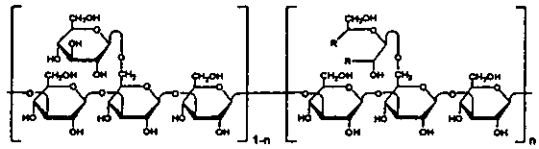
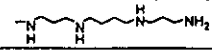
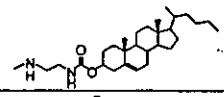
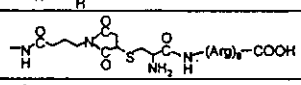
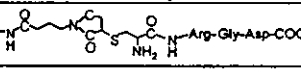


Figure 1. Chemical structure of the chemically modified schizophyllan (SPG) used in this study. The left-hand side shows the intact structure of SPG, and the right shows the modified one. The modification level in Table 1 is defined by "*n*" in the figure. The modification was made by the selective cleavage of 1,2-diol group of the glycosyl side chain with periodate, leading to formyl group formation and subsequently introducing a functional group (see Supporting Information).

Table 1. Sample Codes and the Introduced Chemical Groups

Sample code	R	Modification level[a]	N/P ratio[b]
SP-SPG		4.6 ± 0.3 mol%	0.27
Chol-SPG		6.9 ± 1.0 mol%	0.21
R8-SPG		0.5 ± 0.1 mol%	~0
RGD-SPG		1.3 ± 0.3 mol%	~0

The uptake efficiency of SPG itself is not so high; therefore, we modified SPG with a functional group that can induce passive cellular ingestion. In this work, we introduced spermine (SP), arginine-glycine-aspartic acid tripeptide (RGD), octaarginine (R8), and cholesterol (Chol) (see Table 1). As CpG DNA, we used phosphorothioate 5′-TCCAT*GACGTT*CCTGATG-(dA)$_{40}$-3′ (the immunostimulatory sequence; PuPuCGPyPy is italicized).[12] For a negative control, we used the sequence of 5′-TCCATGA*GCTTC*-CTGAGT-(dA)$_{40}$-3′, where only the CG sequence is reversed (italic) and denoted "non-CpG DNA". In both sequences, a poly(dA)$_{40}$ tail is attached at the 3′ end to increase the complex stability.[7,8] The complexation was carried out by the established method,[7] and the molar ratio (M_{s-SPG}/M_{ODN}) was fixed to 1.5, where M_{s-SPG} and M_{ODN} are the molar concentration of the repeating unit of SPG and

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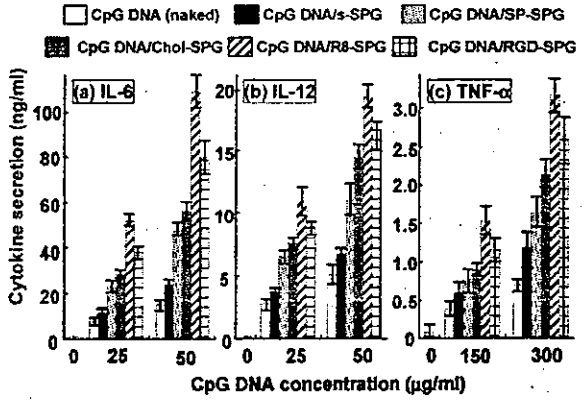

Figure 2. Effect of chemical modification of SPG on CpG DNA-mediated cytokine secretion. The murine macrophage-like cell J774.A1 (1×10^6 cells/mL, 100 μL/well) was stimulated with 25 and 50 μg/mL for IL-6 and IL-12 and with 150 and 300 μg/mL for TNF-α. The amount of secretions was determined with ELISA kits, after incubating the cells in the presence of CpG DNA or its complex for 24 h. The molar ratio ($M_{s\text{-SPG}}/M_{ODN}$) is fixed at 1.5.

CpG DNA, respectively. Although this molar ratio is in excess of SPG over the stoichiometric composition ($M_{s\text{-SPG}}/M_{ODN} = 2/3$),[7] the best antisense effect has been achieved at this composition.[8] Thus, we used this composition in the present work, and the total charge of the complex is maintained at negative at the composition.

When we exposed the murine macrophage-like cell J774.A1 to CpG DNA (complexed or naked), the cytokine secretion increased rapidly and reached a plateau after 9−15 h, being similar to the previous results.[12] After 24 h, all samples showed no increment and seemed to complete the secretion. Therefore, we evaluated the carrier performance from the amount of secreted cytokine after 24 h. Figure 2 plots the averaged amount of cytokine against the CpG DNA dose for three cytokines: IL-6 (a), IL-12 (b), and TNF-α (c), and the experimental variations are indicated by error bars.

When we exposed the cells to naked CpG DNA, the secretions of IL-6, IL-12, and TNF-α are 9.0 ± 1, 2.6 ± 1, and 0.75 ± 0.1 ng/mL, respectively. When the CpG DNA was added as a complex with nonmodified SPG (CpG DNA/s-SPG), the secretion is increased by about 20−40% from the naked assay. This difference should be ascribed to the facts that the complexed CpG DNA neither binds to serum proteins nor suffers hydrolysis from deoxyribonuclease,[11] although SPG itself does not have ability to enter cells. The modified SPG increases the secretion dramatically; it is 5−10-fold compared with the naked assays. Among them, R8−SPG shows the highest performance, RGD−SPG ranks the second, and Chol−SPG follows. The difference between RGD−SPG and Chol−SPG is prominent for IL-6; however, it is relatively small for IL-12 and TNF-α. As far as we know, this is the highest enhancement of the cytokine secretion by carriers.

The ingestion mechanism differs on introduced chemical moieties to the carrier. The cellular membrane is negatively charged, and therefore cations such as spermine can bind to the surface with the Coulombic forces and should be ingested by the regular pinocytosis cycle. This should be the case for SP−SPG; however, ingestion through the electrostatic interaction should not be effective for our case, because the complex is negatively charged in total (see Table 1). Therefore, it interferes with the spermine versus cell interaction. Generally, cholesterol-appended carriers are ingested through LDL receptor and RGD-appended ones, through integrins. Cellular ingestion for these is considered as receptor-mediated endocytosis. Therefore, after ingestion, the RGD−SPG or Chol−SPG/CpG DNA complex is eventually transported to endosome and finally to lysosome, where the compartment pH is kept to about 5 and digestive enzymes are highly activated. The complex releases the CpG DNA because of low pH,[10] and the naked CpG DNA is easily recognized by TLR-9 on the vesicular membrane. This may be a reason for the relatively high secretion for RGD−SPG and Chol−SPG complexes. In contrast with RGD, the arginine-rich peptides such as R8 are seemingly ingested by a different pathway from those of cations RGD and cholesterol, although there is still controversy and little agreement on the uptake mechanism.[13] Some data suggested that the pathway induced by R8 appears to deposit the R8-appended material directly into cytosol. If this were the only pathway to ingest the R8−SPG complex, the cytokine secretion would have been smaller than the others. The precise mechanism to uptake the R8−SPG complex and reason for such enhancement are not clear at this moment.

To sum up the present finding, when SPG is chemically modified with R8, RGD, and Chol and when the CpG DNA complex made therefrom is exposed to macrophages, dramatic enhancement in the cytokine secretion is observed. The secretion increased 5−10 times from the naked dose and 100 times from the background. This performance promises that SPG can be an excellent carrier for CpG DNA.

Acknowledgment. This work was financially supported by the JST SORST program.

Supporting Information Available: Experimental details, materials, SPG chemical modification procedures, cytotoxicity assay for the carriers, assay for non-CpG, confocal microscopy observation, and pH dependence of CD spectrum of the complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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Separation Technique for Messenger RNAs by Use of Schizophyllan/Poly(A) Tail Complexation[1]

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Schizophyllan (SPG) is one of the water soluble β-1,3-glucans and has a peculiar molecular recognition capability, namely, the single stranded SPG (s-SPG) can form a stoichiometric complex with certain polynucleotides such as poly(C) and poly(A), although it cannot bind poly(G) and poly(dC) at all. In this paper, we prepared an s-SPG-appended column and made an attempt to separate polynucleotides on the bases of this molecular recognition capability. The s-SPG-appended column trapped only such RNAs that could form the complex with s-SPG but eluted other RNAs which did not form the complex. Encouraged by the results in the model system, we extended the s-SPG-appended column into separation of native messenger RNAs (mRNAs) from a RNA mixture (total RNA) obtained from yeast. Since eukaryotic mRNAs have a poly(A) tail with 150−300 bases, we supposed that the tails would be trapped by the s-SPG-appended column. The results indicate that mRNAs were separated from total RNA in good yield and with high purity. It should be emphasized that this is the first device to separate natural mRNAs without using a d*A*/d*T* Watson−Crick-type interaction.

Introduction

Sequence analysis of messenger RNAs (mRNAs) is a key element in recombinant DNA techniques and gene engineering, because mRNAs directly code proteins.[2] Therefore, there is a persistent demand for simple and quick separation of pure mRNAs in scientific and industrial fields. The general procedure used for separating mRNAs is based on a method that cellular RNA mixtures (total RNA) are separated from cellular extract by the acid guanidine/phenol/chloroform method,[3] and mRNAs are separated from the total RNA with oligo(dT)-appended devices.[4] Since total RNA contains only 1−2% of mRNAs, a special procedure is necessary to separate such a scarce amount of mRNAs from total RNA. Most eukaryotic mRNAs have a poly(A) tail with 150−300 bases at the 3′ end, and selective recognition of the poly(A) tail thus makes it possible to separate mRNAs.[5−7] Oligo(dT)-appended cellulose columns, magnetic particles, or latex resins are used for this purpose in these days. These techniques can provide highly pure mRNAs, although there are some drawbacks. The oligo(dT) moiety is easily hydrolyzed both in vivo and in vitro and becomes expensive when attached to the device synthetically. If there is an alternative and inexpensive method, it should be very useful in many research fields.

Reagents that can recognize a specific sequence of polynucleotide are of significant importance in the gene engineering. This is usually achieved either by the formation of complimentary hydrogen bonds as in peptide-sustained nucleic acids[8,9] and amide-like *N*-pyrrole oligomers[10,11] or by the electrostatic interaction as in polyethyleneimine,[12,13] and cationic-glycoside.[14,15] As well as these reagents, schizophyllan (SPG) which is also a kind of polysaccharides can be used as a specific polynucleotide-receptor.[16−18] SPG is a natural polysaccharide produced on a large scale by fungus *Schizophyllum commune* of the *Basidiomycota*, and it can be supplied inexpensively (less than 1/1000 of oligo(dT)). The main chain consists of β-(1→3)-D-glucan and one β-(1→6)-D-glycosyl side chain links to the main chain every three glucose residues (Figure 1A).[19,20] In nature, three SPG chains form a triple stranded helix in water, and the helix is dissociated to the single stranded SPG (s-SPG) in dimethyl sulfoxide (DMSO).[21−23] When water is added to the DMSO solution, s-SPG collapses owing to the formation of hydrogen bonds.[24,25] Recently, we found that s-SPG forms a polymer complex with some single stranded polynucleotides with the hydrogen bonding and hydrophobic interactions.[16,17] One of the novel features in this complex is that the complexation occurs in a highly stoichiometric manner: that is, the stoichiometric analysis of the s-SPG/poly(C) complex indicates that two glucose units in the main chain are interacting with one cytosine unit.[17] Atomic force and scanning electron microscopy observations revealed that the complex adopts

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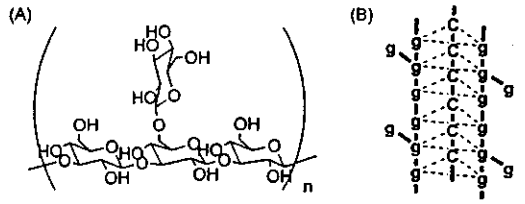

Figure 1. Chemical structure of schizophyllan (SPG) (A), and schematic illustration of the s-SPG/poly(C) complex (B). g and C denote the glucose and cytosine in the chains, respectively, and the dotted lines present the hydrogen bonds.

the same rodlike architecture as the original triple helix.[26] These lines of evidence indicate that the complex consists of the two s-SPG chains and one poly(C) chain to form a new triple helix, as illustrated in Figure 1B. Another novel feature is the specific selectivity of s-SPG for polynucleotides. In the case where homo-polynucleotides are mixed with s-SPG in salt free neutral water, the complexation occurs with poly(C), poly(A), poly(dA), and poly(dT) but not with poly(G), poly(dG), poly(dC), and poly(U).[17] This selectivity is governed by the difference of whether hydrogen-bonding sites of the polynucleotide are free or not. Poly(G), poly(dG), poly(dC), and poly(U) are inactive because of formation of a tetramer or a dimer. The hydrogen-bonding sites in these bases are used to form the intramolecular complex. In contrast, poly(C), poly(A), poly(dA), and poly(dT) do not form such an intramolecular complex and their hydrogen-bonding sites are unoccupied and available for the complexation.

Basic Concept and Strategy to Separate Native mRNAs

Our previous communication[27] reported that an s-SPG-appended column can trap only such RNAs that can form the complex with s-SPG (such as poly(C) and poly(A)) but elutes other RNAs which do not form the complex (such as poly(G)). Eukaryotic mRNAs have a poly(A) tail and we already know that s-SPG can bind poly(A) itself. These facts suggest that one can construct a separation device for mRNA using the s-SPG/poly(A) complexation. Our basic strategy consists of the following procedures (also schematically presented in Figure 2). It is known that to form the complex more than 30 homo-nucleotide sequences are necessary.[28] This means that most natural polynucleotides do not form the complex with s-SPG, except poly(A) tails in mRNA. When total RNA is added into an s-SPG-appended column with an appropriate solvent (loading solvent) which can allow poly(A) tails to bind s-SPG, mRNAs should be trapped by the s-SPG-appended column, whereas ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) should be eluted eventually. After the column is rinsed off to clean residual tRNAs and rRNAs with the loading solvent, only mRNAs should be left in the column. This pure mRNAs can be recovered by rinsing it with a "recovering solvent". This solvent can be hot water whose temperature is higher than the dissociation temperature[17] or an acidic solution in which protonation leads to dissociation of the complex.[29] In this work, we used Tris-HCl buffer (pH 7.5, 0.10 M) at 5 °C as

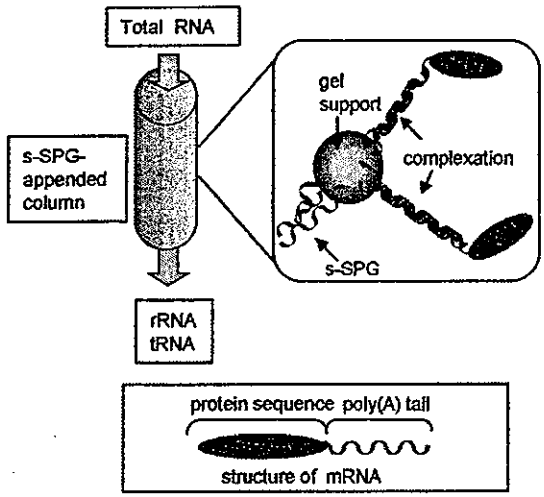

Figure 2. Our basic strategy to separate mRNAs with the s-SPG/poly(A) tail complexation. The s-SPG-appended column contains the gel support on which the s-SPG reducing terminals have been attached to the surface carboxyl groups. Eukaryotic mRNAs have a poly(A) tail and the s-SPG can bind to the tail. When total RNA is added into the column, rRNAs and tRNAs are eluted. The pure mRNAs can be recovered by rinsing with a "recovering solvent".

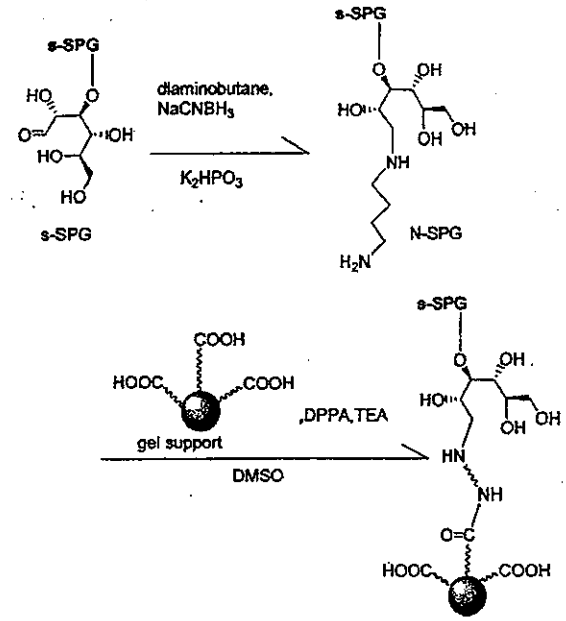

Figure 3. Synthesis scheme of the s-SPG-appended gel support.

the loading solvent and salt free neutral water at 70 °C as the recovering solvent.

Design of an s-SPG-appended column is the most important to avoid nonspecific interactions. To avoid the nonspecific interaction between polynucleotides and cations, we employed a carboxyl carrier gel support. Thus, we successfully introduced an amino group to the reducing terminal of s-SPG (see Figure 3).[30,31] The amino-terminated s-SPG was immobilized on the gel support in the presence of a condensing agent.

Experimental Section

Materials. SPG was kindly supplied by Taito Co. in Japan. The molecular weight and the number of repeating units were

evaluated to be 1.5×10^5 and 231, respectively.[21] Poly(C) and poly(A) were purchased from Amersham Biosciences and poly(G) from Wako Chemicals. Diaminobutane was purchased from Wako Chemicals and diphenyl phosphorazidate (DPPA) from Tokyo Chemical Industry. Sample solution and buffer were made of RNase free distilled water treated with dietyl pyrocarbonate (Nacalai Tesque). Total RNA was extracted from yeast (*saccharomyces cerevisiae* Kyokai No.7). The yeast was cultured in the YM Broth medium (Difco) for 18 h at 30 °C, and the total RNA was isolated by the acid guanidine/phenol/chloroform method using TRIzol reagent (Invitrogen). mRNAs were extracted from total RNA by using PolyATract mRNA Isolation System (Promega) following the instructions from the manufacturer.

Synthesis of Amino-Terminated s-SPG (N−SPG) and Preparation of s-SPG Column. Figure 3 presents our procedure to attach s-SPG chains to a gel support. First of all, we attached diaminobutane to the reducing terminal of s-SPG according to the following procedure. SPG (500 mg, 2.97×10^{-3} mol/glucose) was dissolved in 100 mL of 0.20 M K_2HPO_3 aqueous solution. Diaminobutane (100 mg, 1.13×10^{-3} mol) and sodium cyanoborohydride (100 mg, 1.59×10^{-3} mol) were added to the SPG solution four times every 6 h. Then the mixture was stirred for 26 h at 30 °C. After the mixture was dialyzed for 2 days with distilled water, the solvent was removed by a freeze-drying method. The introduction of diaminobutane into s-SPG was confirmed qualitatively by the ninhydrin reaction on a thin-layer chromatography plate. The diaminobutane-attached s-SPG was denoted by N-SPG.

N-SPG was reacted with carboxyl groups on a gel support with a coupling reaction using DPPA in DMSO as follows. AF-Carboxyl TOYOPEARL 650M (Tosoh) (18 g) was suspended into 100 mL of dehydrated DMSO. The mixture containing N-SPG (200 mg, 1.19×10^{-3} mol/glucose), triethylamine (45 mg, 4.44×10^{-4} mol), and DPPA (250 mg, 9.08×10^{-4} mol) was shaken for 48 h at 30 °C. Then the mixture was filtrated and the residue was washed with DMSO (50 mL x 3) and distilled water (50 mL x 3). The resultant s-SPG-appended gel support (9 mL) was packed into a plastic column with 15 mm in diameter and 40 mm in length (s-SPG column). As a reference, AF-Carboxyl TOYOPEARL 650M was packed into a column with 15 mm in diameter and 40 mm in length (blank column). It was difficult to estimate the amount of immobilized s-SPG on the gel support by the elemental analysis, because the mass fraction of the gel support is more than 99% and it is practically impossible to detect the N atom fraction.

Instead of the elemental analysis, we examined how much poly(C) was bound onto the gel support. According to our work,[17] poly(C) has the highest binding constant for s-SPG and the complexation takes place with the highest yield among polynucleotides. Therefore, we can measure (or have a semiquantitative value at least) how much s-SPG molecules are bound onto the support. The s-SPG column was washed in an excess amount of poly(C) solution under the loading conditions, and after 12 h, free poly(C) was removed by rinsing off the column with the loading solvent. The rinse of the column was repeated until there was no poly(C) detected by the UV measurement. The bound poly(C) was collected by rinsing the recovering solvent. In this experiment, the amount of poly(C) bound to the s-SPG column is estimated to be 8.2×10^{-6} mol/nucleotide from the UV measurement. If the stoichiometry (two glucose units in the s-SPG main chain with respect to one cytosine) is held even on the gel surface in the s-SPG column, the amount of s-SPG which is available for trapping RNAs on the gel is calculated to be 2.4×10^{-6} mol/glucose unit per 1 mL-gel(wet).

Separation of RNAs with s-SPG Column. After the s-SPG column was washed with the loading solvent, the solvent was exchanged for the RNA solution at 5 °C. The concentrations and volumes of the RNA solution were 1.25×10^{-3} M/nucleotide and 200 μL for poly(A) or poly(C), 7.09×10^{-5} M/nucleotide and 200 μL for mRNAs, and 2.88×10^{-4} M/nucleotide and 500 μL for total RNA. The elutes were monitored by following the absorbance at 260 nm on a UV-2200A UV−vis spectrophotometer (Shimadzu) with a 5.0 × 10 mm quartz cell.

Northern Blot Analysis of mRNAs. A total of 1.0 μL of RNA solutions fractionated were spotted onto a Hybond-N^+ nylon membrane (Amershame Biosciences). Oligo$(dT)_{18}$ was purchased from Bio Labs as a hybridization probe for mRNAs. Digoxigenin labeled oligo$(dT)_{18}$ (Dig-oligo(dT)) was prepared by using a DIG oligonucleotide tailing kit (Roche Diagnostics) according to the supplier's instructions. Dig-oligo(dT) was hybridized with mRNAs on the membrane for 12 h at 25 °C. mRNA/Dig-oligo(dT) hybrid was treated with an alkaline phosphatase-labeled Dig-antibody (Roche Diagnostics). The mRNA/Dig-oligo(dT)/alkaline phosphatase-labeled Dig-antibody ternary hybrid on the membrane was detected by using 4-nitro blue tetrazolium chloride (Roche Diagnostics) and 5-bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostics).

Electrophoresis. SeaKem GTG agarose gel (Cambrex) was prepared at 1.5 wt % concentration with MOPS buffer, and formalin was electrophoresed for 25 min at 100 V. The migrants were stained with ethidium bromide. The migration patterns were revealed by UV irradiation and analyzed on a Typhoon 9200 phosphorimager (molecular dynamics, Amersham Pharmacia Biotech).

Reverse Transcription-PCR (RT-PCR). A total of 9.0 μL of RNA solution was reverse-transcribed into complimentary DNA (cDNA) using Thermo Script RT-PCR System (Invitrogen) with oligo$(dT)_{18}$ primer. Then 2.0 μL of the reverse transcription reactant was utilized for PCR amplification of cDNA fragments for TDH1 (sense primer, 5′-GCTATTAACGGTTTCGGTAGA-3′; antisense primer 5′-TCTACGGCAACAGAGGAG-3′; 833 bp PCR product) and ACTYEAST (sense primer, 5′-CTTCCCATCTATCGTCGGTAG-3′; antisense primer 5′-TCTTATGCTGCTTTCACCAGG-3′; 1011 bp PCR product). The PCR reaction mixture contained 2 units of Taq DNA polymerase, PCR buffer, 2.0×10^{-4} M dNTP, 1.0 μL of sense and antisense primers in a total volume of 50 μL. PCR was performed for 40 cycles. Each cycle involved a 94 °C/1min denaturation step, a 55 °C/1min annealing step, and a 72 °C/1min polymerization

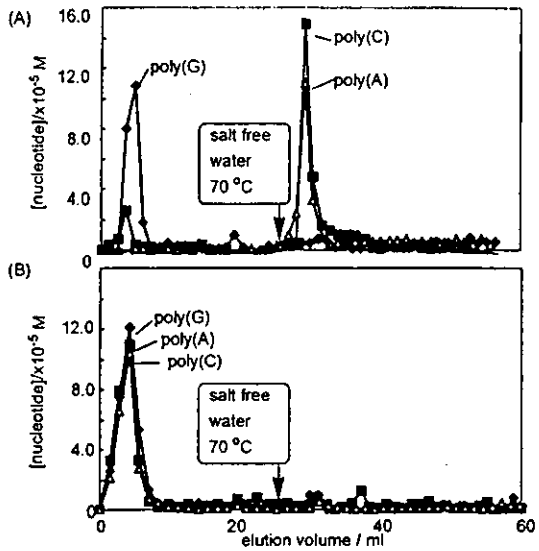

Figure 4. Comparison of the elution curves of homo-polynucleotides from the s-SPG column (A) and the blank column (B); poly(C) (■), poly(A) (△), poly(G) (◆).

step. The PCR products were electrophoresed on agarose gel and stained with ethidium bromide.

Results and Discussion

Molecular Recognition/Separation with s-SPG Column in Model Systems. Figure 4A compares elution curves among poly(C), poly(A), and poly(G) when they were loaded and subsequently recovered. As mentioned above, poly(C) and poly(A) can form the complex, but poly(G) cannot. As expected from these facts, elution of poly(C) or poly(A) was not observed under the loading conditions, and once the solvent was changed to the recovering conditions, they were eluted immediately. On the other hand, poly(G) was not trapped under the loading conditions. Figure 4B shows the elution curves when the exactly same polynucleotides and solvents were used for the blank column, to which no s-SPG had been attached. The column did not trap any polynucleotide under the loading conditions. These results indicate that poly(C) and poly(A) are retained in the s-SPG column through the complexation, not owing to nonspecific interactions such as ion-pair formation or physical adsorption. When we calculated the amount of the recovered poly(C) and poly(A), they were 93 and 97% of the fed polynucleotides, respectively. One can regard, therefore, that virtually 100% of poly(A) and poly(C) can be trapped in the column under the loading conditions and the recovering conditions can elute all bound polynucleotides.

We carried out the similar experiment under the same conditions as Figure 4, using a pure mRNAs sample obtained from yeast. Figure 5A plots the elution curve and panel B shows the northern dot blot for each fraction numbered in panel A. Although the S/N ratio is larger than that in Figure 4 owing to the lower concentration of mRNAs, there is no peak in the loading regime and a large absorption peak is clearly observed after changed to the recovering conditions. Panel B shows that this large peak contains mRNA at high concentrations. The present results indicate that the mRNAs

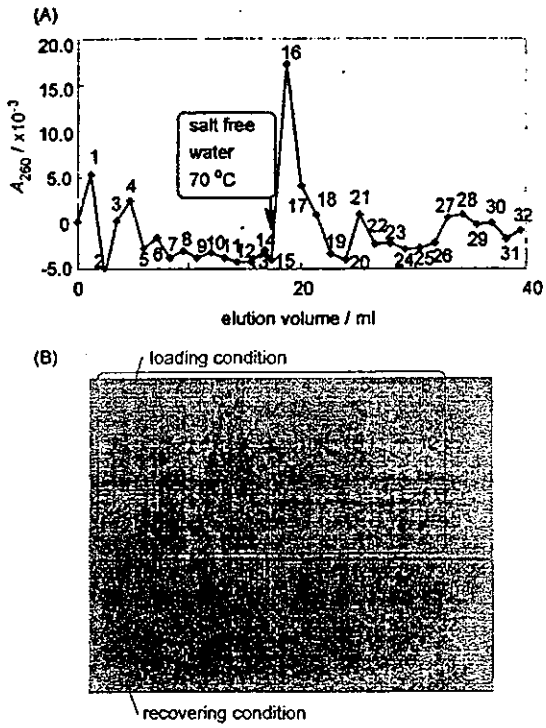

Figure 5. Elution curve of mRNAs from the s-SPG column (A) and the northern dot blot for all of the fractions (B).

elution is only observed for the 16−18th fractions. This result confirms that mRNAs can be trapped and recovered similarly to poly(A) and poly(C). The recovery rate is calculated to be about 90%.

The above two model experiments prove that our basic concept is valid and the s-SPG column can recognize the polynucleotides that can bind s-SPG in solution. Generally, one mRNA molecule consists of more than a few thousands bases and the poly(A) tail is just a small fraction of it. The present finding establishes that this tail length is sufficient for the s-SPG column to trap mRNAs.

Separation of mRNAs from Total RNA. Figure 6 shows the elution behavior of total RNA. Most of RNAs (more than 95%) are eluted immediately under the loading conditions. This feature is expected from the fact that total RNA contains only 1−2% of mRNAs and the residual components are rRNA and tRNA, which would not be trapped by the s-SPG column. After changed to the recovering conditions, a small amount of RNA is eluted. The northern dot blot (Figure 6, panel B) shows that mRNAs are not included in the major fraction at all but are concentrated in the 18th fraction. For comparison, Figure 7A shows the elution behavior for the blank column. The elution curve itself in the blank column exhibits the same feature, that is, the major elution in the 3/5 fractions and the minor elution after changed to the recovering conditions. However, a remarkable difference is observed for the northern blot analysis. In the blank column, mRNAs are included in the major fraction and the minor elution does not contain any mRNAs (Figure 7, panel B). This experiment demonstrates that the s-SPG column can separate a trace amount of mRNAs from total RNA owing to the s-SPG/mRNA complexation.

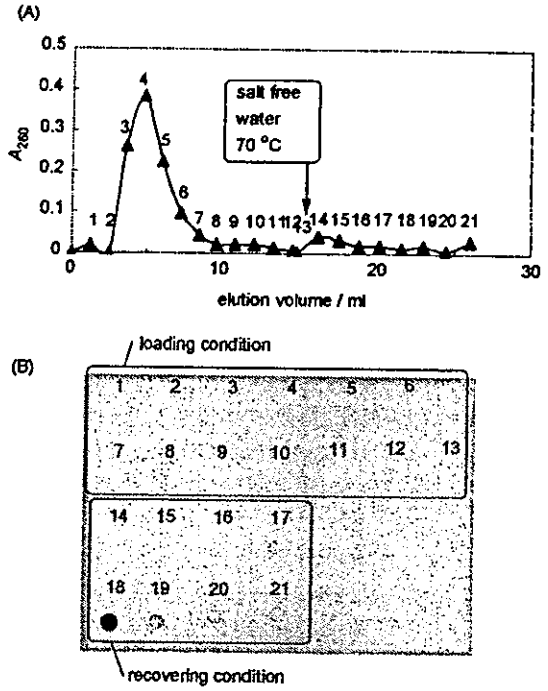

Figure 6. Elution curve of total RNA from the s-SPG column (A) and the northern dot blot for all the fractions (B).

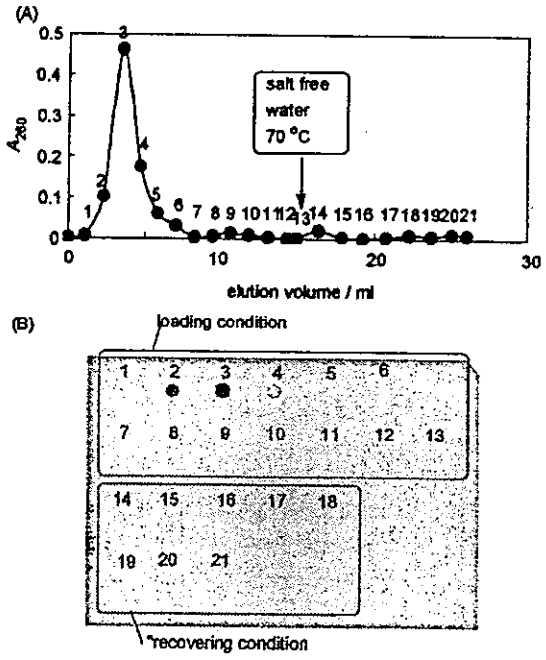

Figure 7. Elution curve of total RNA from the blank column (A) and the northern dot blot for all the fractions (B).

Purity of the Separated mRNAs Sample. Since mRNAs have a large distribution in the number of base pairs, the gel electrophoresis band is smeared, ranging from approximately 8.0 kb to 0.5 kb. On the other hand, 28 S rRNA, 18 S rRNA, and 5S rRNA appear as single clear bands, respectively.[32] Purity of the mRNAs separated by the s-SPG column was examined by agarose gel electrophoresis. Figure 8A compares the migration patterns between the total RNA and mRNAs samples. The pattern of total RNA in lane 1 is a typical one, having three 28 S rRNA, 18 S rRNA, and 5S

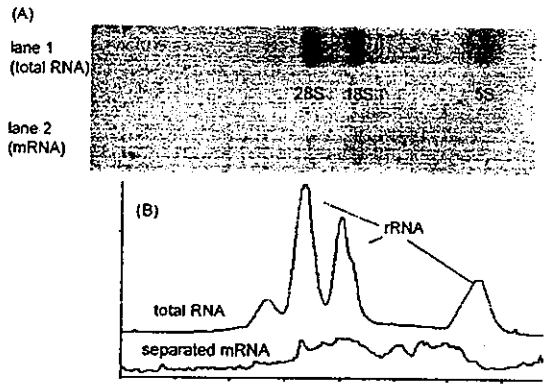

Figure 8. Comparison of the electrophoresis pattern among total RNA (lane1) and mRNAs separated by the s-SPG column (lane 2) (A) and the intensity profiles of lane 1 and lane 2 (B).

rRNA bands as indicated in the figures. On the other hand, when we examined the separated mRNAs, it showed a smear and weak band as presented in lane 2.

Using a densitometer, the intensity profiles of the lane 1 and lane 2 are plotted in Figure 8B. In the profile of lane 2, we can recognize small peaks due to rRNAs, overlapping with the mRNAs broad band. Applying a peak de-convolution technique, the peaks of rRNAs were subtracted from the original peak and the purity of mRNAs was estimated to be 87%. In these days, the oligo(dT)-appended latex deposited on the spin-column has been commercialized as a mRNAs separation kit. The application of this kit according to the recommended recipe to the present system showed that the purity of isolated mRNAs is 66−79%. It indicates that the s-SPG column can separate mRNAs in higher purity than the oligo(dT)-appended latex system.

We demonstrated to confirm that the separated mRNAs could be used for RT-PCR. If most of the isolated mRNAs are not cleaved in the separation process, the mRNAs should be reverse-transcribed into cDNAs by using the oligo$(dT)_{18}$ primer. Then, it would be possible to amplify an optional sequence from the cDNAs mixture. In this work, ACTYEAST (1011 bp) and TDH1 (833 bp) sequences were amplified in the cDNAs mixture. Since ACTYEAST and TDH1 are expressed constantly in a yeast cell, they can be regarded as a representative system of all mRNAs. Figure 9 shows the electrophoresis pattern of the RT-PCR products from the separated mRNAs sample. It is clearly seen from Figure 9 that the single DNA product of about 1000 base pairs appears in both experiments of ACTYEAST and TDH1. These results support the view that the mRNAs separated in the s-SPG column is available for gene engineering.

Conclusions

In this paper, the s-SPG-appended column was prepared and applied to a separation system containing various RNAs. We demonstrated that the s-SPG column can trap only RNAs which form the complex with s-SPG. Also important is the finding that natural mRNAs are trapped in the s-SPG column by the s-SPG/poly(A) tail complexation. This result proves that s-SPG can form the complex not only with artificial homo-polynucleotides but also natural mRNAs. Furthermore,

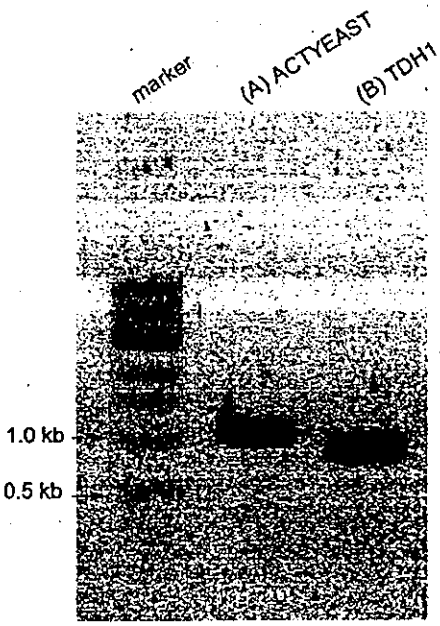

Figure 9. Electrophoresis patterns of RT-PCR products from mRNAs separated by the s-SPG column. ACTYEAST sequence (A), TDH1 sequence (B).

we succeeded in separating mRNAs from total RNA by using this s-SPG-appended column. This fact indicates that s-SPG/mRNA complexation occurs with high selectivity even in the RNA mixture. It should be emphasized that this separation system consists of only a neutral polysaccharide, but not of nucleobases at all. As far as we know, this is the first example to recognize and separate natural mRNAs by using polysaccharide-polynucleotide complexation.

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Physicochemical Properties and Bio-degradation of Segmented Polyurethane and Poly(urethane-urea) Derived from Lysine-Based Diisocyanate

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Segmented polyurethanes (SPU) and poly(urethane-urea)s (SPUU) which were expected to yield non-toxic degradation products were synthesized from lysine-based diisocyanate (LDI), 1,3-propanediol (PDO), 1,4-butanediamine (BDA), and polycaprolactone diol (PCL). SPU and SPUU were synthesized via a standard two-step prepolymer method. The hard segment fraction was changed in order to tune the mechanical properties and the degradability. The aggregation structures of the SPU and SPUU were characterized by IR spectroscopy and DSC. IR spectroscopy revealed that fraction of hydrogen bonded urethane and urea carbonyl groups increases with an increase in hard segment fraction. DSC thermogram revealed that the glass transition temperature of BDA-based hard segment is higher than that of PDO-based one. Tensile tests revealed the excellent elastic properties of PCL(1250)(71)BDA. Furthermore, the degradation of SPU and SPUU were investigated by exposing the polymers to buffer solution at 310 K (pH=7.6). The degradation rate of SPU increased with an increase in soft segment fraction. This is because the soft segment has the hydrolyzable ester linkages and is susceptible to hydrolysis compared with that of the urethane linkage. Finally, an electrospray deposition method was used to fabricate biodegradable SPU micro-fibers. FE-SEM images showed that higher concentration of solution favored to form of uniform biodegradable micro-fibers without beads-like structure.

Key words: Degradation, Lysine-based diisocyanate, Polyurethanes, Poly(urethane-urea)s, Electrospray Deposition

1. INTRODUCTION

The use of degradable polymers in tissue engineering for replacement or repair of wide range of tissues is an area of intensive research in recent years. A variety of biodegradable polymers have been developed in the last two decades. However, the majority of these polymers are typically hard and brittle plastic and few biodegradable elastomeric polymers have been synthesized.[1]

The recent development of diisocyanates based on lysine has removed an obstacle to synthesizing biodegradable elastomers expected to yield non-toxic degradation products.[2] It was surmised that if the diisocyanate were liberated by hydrolysis of the urethane bonds of the polymer during degradation the isocyanate functionalities would react with water to regenerate the lysine derivative, an essentially non-toxic products.

Recently, there has been growing interest in a fiber production technology known as electrospray deposition (ES). ES is unique as a fiber spinning process because it can change the morphology and diameter depending on the processing parameter such as solution concentration and applied electric field strength.[3] ES can produce highly porous non-woven fabrics consisting of well-defined fibers which are used for tissue engineering.

In this study, the segmented polyurethanes (SPU) and segmented poly(urethane-urea)s (SPUU) were synthesized from lysine-based diisosyanate. The physical and structural characterizations of SPU and SPUU were performed using differential scanning calorimetry and IR spectroscopy. Then the mechanical properties were investigated by stress-strain measurement. The degradation behavior of SPU and SPUU were also investigated. ES technique was used to fabricate biodegradable fibers. The morphology of electrospray deposited fiber was investigated by field-

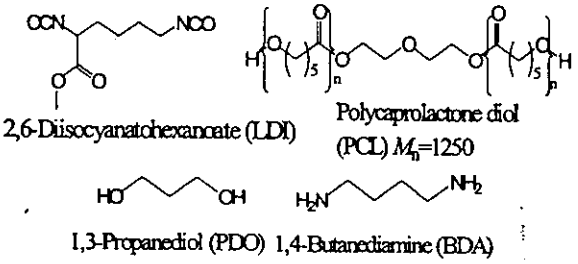

Figure 1 Chemical structures of diisosyanate, soft segment polyol, and chain extenders.