in the degree of polymerization), respectively. Poly(A) and poly(C) were purchased from Amersham Pharmacia (Buckinghamshire, UK) and the degree of polymerization, calculated from the reported sedimentation velocities [17], are 320 and 570, respectively. RNase free, deionized, and distilled water, spectroscopic grade DMSO, NaCl, and KCl were purchased from Wako Chemical (Osaka, Japan). A polynucleotide sample was mixed with s-SPG by adding a s-SPG/DMSO solution to a polynucleotide/water solution. Hereinafter, we denote the mixture as poly(X) + s-SPG, where X can be A or C. The water volume fraction in the water/DMSO mixture (V_w) was fixed at 0.92 for all measurements. The molar concentrations of s-SPG ($M_{s\text{-}SPG}$) and the polynucleotide ($M_{poly(X)}$) in the DMSO/water mixture were 1.0×10^{-3} and 2.6×10^{-4} M, respectively. The apparent pH (pH*) of the DMSO/water solutions was controlled by adding NaOH and HCl aqueous solutions, and monitored with a Horiba D-22 pH meter at room temperature. All the samples were kept at 5 °C for at least 3 days before the measurement.

Circular dichroism (CD) in 230–320 nm region was measured on a Jasco J-720WI spectropolarimeter with a 1.0 cm cell equipped with a water-jacket to control the cell temperature. The measurements were carried out over the pH* range 4–10, the salt concentration range 0–0.3 M, and the temperature range 5–70 °C. The molecular ellipticity ([θ]) was determined by conventional methods [14] and the [θ] value at the peak top or bottom position was denoted as $[\theta]_{max}$.

3. Results and discussion

3.1. The apparent pH dependence of complexation

Fig. 2 compares the CD spectra between poly(C) and poly(C) + s-SPG at pH* 4, 7, and 10 (from top to bottom). The CD spectra of two solutions (i.e., polynucleotide itself and its mixture with s-SPG) are identical at pH* 4, but significantly different from each other at pH* 10 and 7. Furthermore, the poly(C) spectrum at pH* 4 is different from those of poly(C) at pH* 10 and 7, suggesting the presence of a transition between pH* 4 and 7. Fig. 3 presents the CD spectra of poly(A) and poly(A) + s-SPG at pH* 4, 7, and 10. Similar to the poly(C) system, the spectra of poly(A) and poly(A) + s-SPG are identical at pH* 4 and different at pH* 7 and 10, and the poly(A) spectrum changes with increasing pH* from 4 to 7.

The s-SPG sample has no absorption in the 230–300 nm region where the CD measurements were carried out. Therefore, the CD spectrum in this region is related to chirality of the polynucleotide chains, particularly to how the base moieties stack in the helix [16]. Previously, we examined the CD spectra for the poly(A) and poly(C) systems at pH* 7 and found that the difference between polynucleotide itself and its mixture can be ascribed to the fact that the polynucleotide and s-SPG form a macromolecular complex [13–15]. Since each spectrum at pH* 10 for the mixture is identical to that of the corresponding sample at pH* 7, the complex is maintained at pH 10. At pH* 4, there is no difference between the polynucleotide itself and the mixture, indicating that s-SPG cannot form a complex with the polynucleotides at pH* 4.

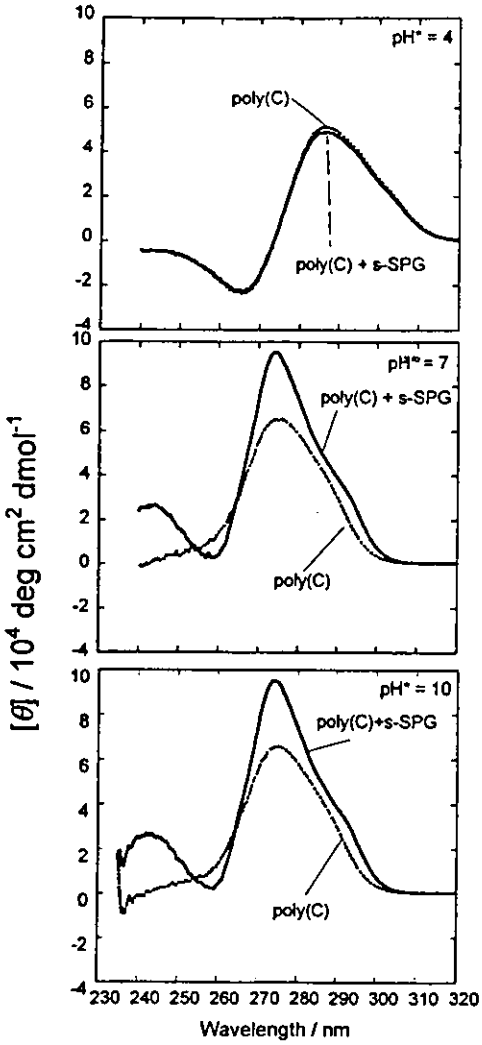

Fig. 2. The apparent pH dependence of the CD spectra for poly(C) (dotted lines) and its mixture with s-SPG (solid lines) measured at 10 °C.

Fig. 4 plots the $[\theta]_{max}$ against pH* for the poly(C) and poly(A) systems. The $[\theta]_{max}$ values for the polynucleotides (before mixing, indicated by filled circles in the figure) seem constant between pH* 10 and 7.0. At pH* 6–7, $[\theta]_{max}$ drastically changes and becomes almost constant at pH* values <6 for poly(A) and poly(C). This feature indicates that the polynucleotides themselves (before mixing) undergo a conformational change at pH* 6–7. According to the literature [16,18], poly(C) and poly(A) adopt a single strand in the neutral and basic regions and a double strand in the acidic region. The mixtures show different $[\theta]_{max}$ from those of polynucleotides themselves in the pH* range 6.5–10 and a drastic change in CD at the same pH* where polynucleotides themselves show the transition. In the pH* range 4–6, the polynucleotide itself and the mixture show the same $[\theta]_{max}$ values, indicating no interaction between s-SPG and the polynucleotide. This feature clearly indicates that the conformational change of each polynucleotide is related to the dissociation/association of

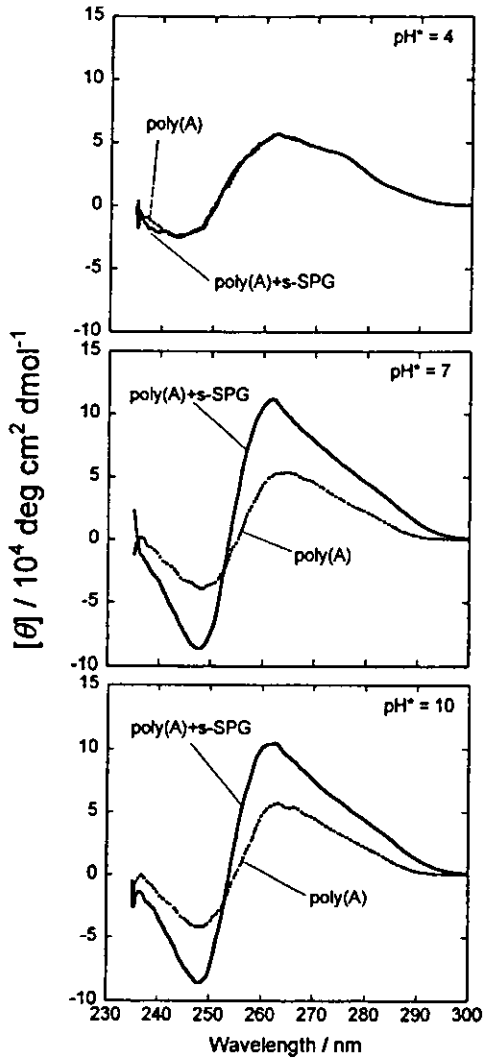

Fig. 3. The apparent pH dependence of the CD spectra for poly(A) (solid lines) and its mixture with s-SPG (dotted lines) measured at 10 °C.

the complex: the single strand of the polynucleotides can form complex, whereas the double one cannot.

Previous work [18] shows that dimerization of the nucleic acids is the origin to form the double stranded conformation at pH* 6–7. Fig. 5 presents the proposed dimer structure for the cytosine and adenine residues [18]. Incremental increases in the hydrogen-ion concentration induces protonation of the base and hydrogen bonds are formed between two bases, which results in the conformational transition of the polynucleotide chain from the single to double strand. It is well known that the pK_a is 4.2–4.6 for N_3 in cytosine and 3.5–3.9 for N_1 in adenosine, respectively [19]. These reported values are lower than those obtained for poly(C) and poly(A). Hartman and Rich [18] studied the titration curve for poly(C) and confirmed that protonation of poly(C) takes place at pH* 6–7. The difference in pK_a between the monomeric and polymeric systems can be explained by a neutralization effect where "the positive

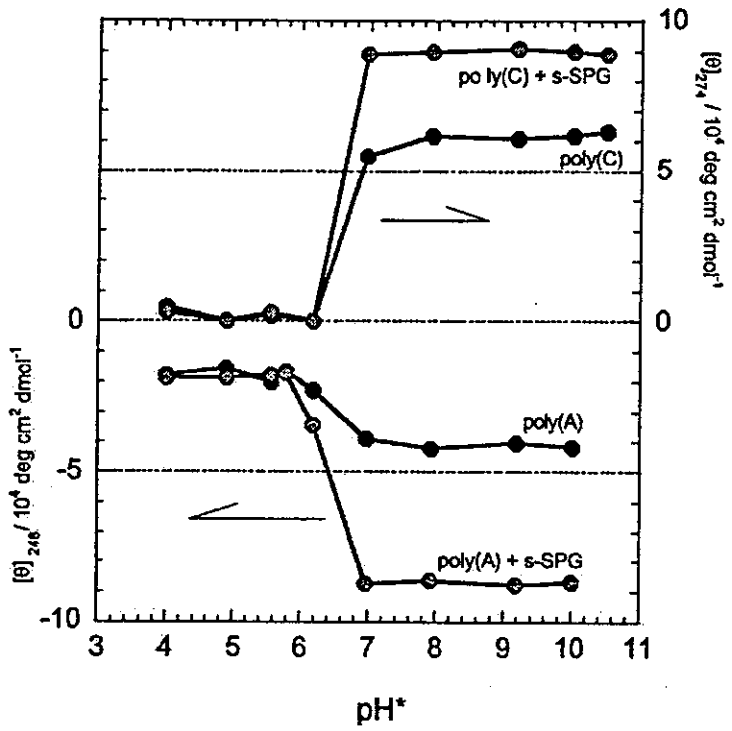

Fig. 4. Comparison of pH* dependence of $[\theta]_{max}$ for the poly(A) system and poly(C) system. For convenience to compare the transition, $[\theta]_{274}$ for the poly(C) is plotted in the upper part and $[\theta]_{248}$ (see Fig. 3, this is the negative band) is plotted in the lower part.

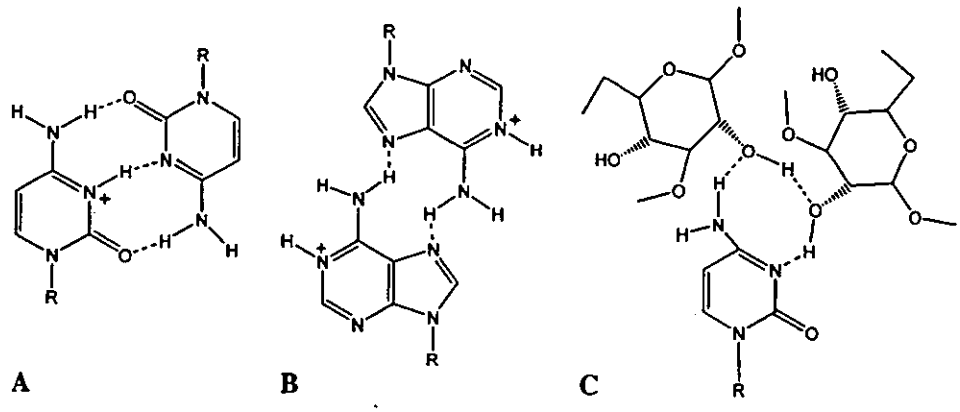

Fig. 5. The hydrogen bonding schemes between cytosine residues in the acidic solution (A), between adenine residues (B), and a proposed model for the complex binding site made of two glucose and one cytosine residues.

charge on the base dimer partially neutralizes the two negative charges on the ribose phosphate chains" [18]. Since this effect can be generally extended to the poly(A) system, we consider that pK_a of poly(A) also shifts from 3.5–3.9 (monomer) to 6.0. Therefore, we confirm that the protonation and the subsequent conformational change are responsible for the dissociation of the complex.

The preceding discussion suggests that there are two possible causes leading to the dissociation of the s-SPG/polynucleotide complex. One cause is the loss of the hydrogen bonding site for s-SPG. According to our proposed model for the complex, it consists of two s-SPG chains and one polynucleotide chain and these chains interact with each other through the hydrogen bonding as shown in Fig. 5C. Since the dimerization of cytosine uses the same nitrogen atoms (N_3 and N_4) as those involved in the complexation, s-SPG loses a binding site after cytosine undergoes dimerization, resulting in dissociation of the complex.

The second cause for the dissociation is the dissimilarity in the helix parameters between the complex and the polynucleotide duplexes. As mentioned in Section 1, similarity in the helix parameters between the complex and the polynucleotides can explain the complexation. According to the literature [16,18], the helix parameters for the double strand of poly(A) and poly(C) are a right-handed 8_1 helix with a 30.4 Å pitch (3.8 Å for the axial rise per residue) and a right-handed 11_1 helix with a 37.3 Å pitch (3.4 Å for the axial rise per residue), respectively. In both cases, the axial rise per residue is considerably larger than that of the single strand and t-SPG. We suggest that this enlarged residue distance makes it difficult for s-SPG to form a complex with the polynucleotides in the acidic region.

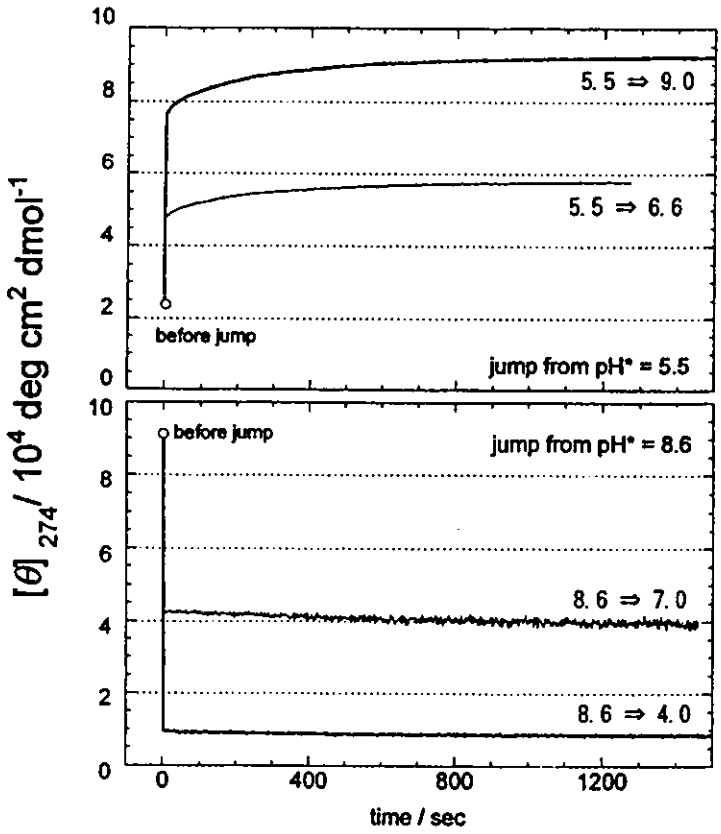

Fig. 6. Time course of $[\theta]_{274}$ after pH* jumped from the acid to base region (complexation) in the upper panel and form the base to acid region (decomplexation) in the lower panel for the poly(C) + s-SPG system.

Fig. 6 presents the time course of $[\theta]_{274}$ after changing pH* from the basic to the acidic region (complexation) in the upper panel, and from the acidic to the basic region (decomplexation) in the lower panel. The data indicate that the complexation and decomplexation take place reversibly. After changed pH*, $[\theta]_{274}$ reaches the equilibrium value immediately for the decomplexation, while, it takes about 1000 s for complexation. This difference can be explained by the fact that diffusion of both chains is the rate determining step for the complexation.

4. The salt concentration dependence of complexation

Fig. 7 compares the CD spectra between a neutral solution without salt and a neutral solution containing 0.3 M NaCl. Poly(C) shows a substantial increase in the CD intensity with the increasing salt concentration. This observation can be explained by the fact that higher salt concentration enhances the electrostatic shielding, which, in turn, causes an increase in the number of the stacked bases in the helix. On the other hand, the spectrum for the complex seems independent of the salt concentration, confirming that most of the bases are already stacked in the complex [14]. Fig. 8 compares the melting curves when the NaCl concentration is changed from 0 to 0.3 M. The melting temperature (T_m) is determined, using conventional methods ([13,14]), and plotted against the salt concentration for both NaCl and KCl in Fig. 9.

The melting temperature drastically increases at low concentrations, reaches the maximum around 0.07 M, and gradually decreases with further increases in the salt concentration. It is interesting that NaCl and KCl give different T_m, suggesting that a specific interaction between poly(C) and these metal cations is involved. Cations

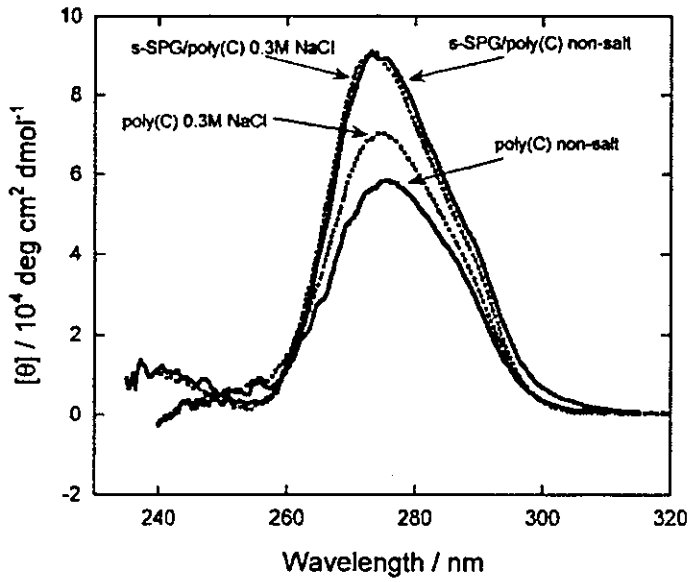

Fig. 7. NaCl concentration dependence of the CD spectra for poly(C) and poly(C) + s-SPG measured at 10 °C. The pH* was controlled by 10 mM Tris buffer.

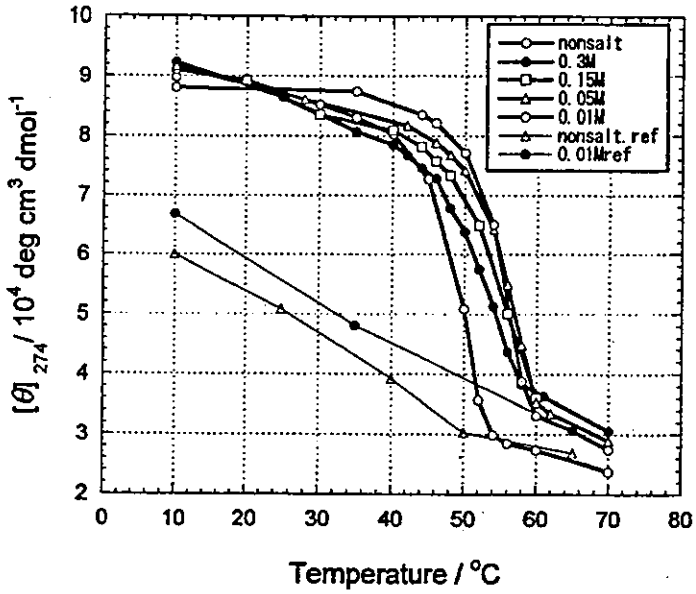

Fig. 8. NaCl concentration dependence of the melting curves of the poly(C) + s-SPG complex. The pH* was controlled by 10 mM Tris buffer. For comparison, the temperature dependence of $[\theta]_{274}$ for poly(C) solutions are presented.

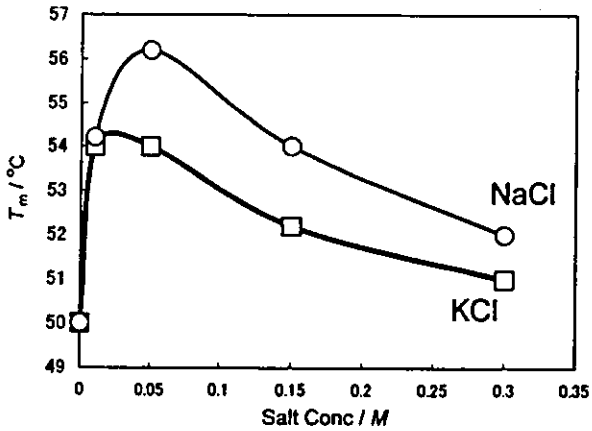

Fig. 9. Comparison of the salt concentration dependence of T_m between NaCl and KCl.

preferably bind to the phosphate anion in polynucleotides and the phosphate anions in polynucleotides interact with the base and ribose through water molecules [16]. Therefore, the increment of T_m at lower salt concentrations may be ascribed to the fact that the cations bound to the phosphate stabilize hydrogen bonding. At higher salt concentrations, the electrostatic shielding effect can stabilize the poly(C) helix as shown in Fig. 9. Hence, the free energy change resulting from decomplexation becomes small, which lowers the T_m. This mechanism might explain the decrease in T_m with increasing the salt concentrations.

5. Conclusions

The present paper demonstrates that formation of the complex between s-SPG and single-stranded RNAs as well as its stability strongly depend on pH and the ionic strength. The complex is formed in the pH* range 6.5–10 and dissociated in the pH* range 4–6. Both poly(A) and poly(C) are double stranded in the pH* range 4–6 and a single strand in the pH* range 6.5–10. Along with this conformational transition, dissociation and complexation take place reversibly. The T_m reaches the maximum around 0.05 M of NaCl and KCl and the value of T_m depends on the nature of the cation species. This observation suggests that some specific interaction is involved in stabilization of the complex.

Acknowledgments

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References

[1] K. Tabata, W. Ito, T. Kojima, S. Kawabata, A. Misaki, Carbohydr. Res. 89 (1981) 121–135.
[2] T. Yanaki, W. Ito, K. Tabata, T. Kojima, T. Norisuye, N. Takano, H. Fujita, Biophys. Chem. 17 (1983) 337–342.
[3] S. Young, R.R. Jacobs, Carbohydr. Res. 310 (1998) 91–99.
[4] T. Norisuye, T. Yanaki, H. Fujita, J. Polym. Sci., Polym. Phys. Ed. 18 (1980) 547–558.
[5] T. Norisuye, T. Yanaki, Polym. J. 15 (1983) 389–396.
[6] K. Yanaki, T. Norisuye, H. Fujita, Macromolecules 13 (1980) 1462–1466.
[7] T. Sato, K. Sakurai, T. Norisuye, H. Fujita, Polym. J. 16 (1983) 559–567.
[8] T. Itou, A. Teramoto, M. Matuo, H. Suga, Carbohydr. Res. 160 (1987) 243–257.
[9] B.T. Stokke, A. Elgaster, D.A. Brant, S. Kitamura, Macromolecules 24 (1991) 6349–6351.
[10] T.M. McIntire, D.A. Brant, J. Am. Chem. Soc. 120 (1998) 6909–6919.
[11] S.H. Young, W.J. Dong, J. Biol. Chem. 275 (16) (2000) 11874–11879.
[12] G.G. Martin, G.C. Cannon, C.L. McCormick, Biomacromolecules 1 (2000) 49–60.
[13] K. Sakurai, S. Shinkai, J. Am. Chem. Soc. 122 (2000) 4520–4521.
[14] K. Sakurai, M. Mizu, S. Shinkai, Biomacromolecules 2 (2001) 641–650.
[15] Sakurai et al., in preparation.
[16] W. Saenger, Principles of Nucleic Acid Structure, Springer, New York, 1984.
[17] Amersham Pharmacia, Technical data report, 2001.
[18] K.A. Hartman, A. Rich, J. Am. Chem. Soc. 87 (1965) 2036–2039.
[19] G.D. Fasman (Ed.), Handbook of Biochemistry and Molecular Biology, vol. I., Nucleic Acids. Chem. Rubber Co., OH, 1975.

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Lactose-appended schizophyllan is a potential candidate as a hepatocyte-targeted antisense carrier†‡

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A schizophyllan (β-1,3-glucan) derivative carrying lactose-appendages prepared by reductive amination can form stable macromolecular complexes with polynucleotides, shows excellent affinity with a lactose-binding lectin, and effectively mediates gene transfection into hepatocytes.

Over the past decade, researchers in the area of supramolecular chemistry have achieved impressive progress towards the development of efficient antisense delivery systems for clinical therapy.[1] To further develop these delivery systems with the minimum side effects and reduced cost, carriers should show the following properties: (1) easy preparation of carrier/antisense complexes, (2) low cytotoxicity, (3) no immunogeneity and (4) high selectivity for target cells. Artificial carriers reported so far include poly-L-lysines,[2] polyethyleneimines,[3] polyamidoamine dendrimers,[4] *etc.*, which mostly utilize electrostatic interactions to form carrier–antisense complexes.

Schizophyllan (SPG), β-1,3-glucan, has been of great interest for many researchers because of its gel-forming ability as well as its anticancer activity.[5] Physical and structural studies have revealed that SPG exists in a unique triple-stranded helical structure (t-SPG) in aqueous solution, whereas it is dissociated into the individual single strands (s-SPG) in dimethyl sulfoxide (DMSO).[6] Recently, we found that unique triple-stranded macromolecular complexes are formed when s-SPG in DMSO is mixed with polynucleotides in aqueous solution (Fig. 1).[7] This finding encouraged us to develop new SPG-based antisense carriers, in which carrier–antisense complexes are formed by the unique 'shape fitting' between two helical components (SPG and polynucleotide), instead of the conventional electrostatic interactions. In the series of our research, we found several advantages of the macromolecular complexes as antisense carriers over the conventional ones: (1) the macromolecular complexes are stable under physiological conditions, (2) complexed polynucleotides are protected against degradation by DNase[8] and (3) the complexed polynucleotides can be quickly released in the presence of the complementary RNA. In addition to the expected long blood-circulation time arising from the lack of β-1,3-glucanase in human body, these characteristics suggest the potential utility of SPG to mediate antisense transfer in human cells. Our next step should be, therefore, to develop a *cell-targeted* carrier based on SPG. In this respect, SPG-derivatives carrying oligosaccharide appendages are of great interest, since oligosaccharides can act as specific ligands for carbohydrate-binding proteins (lectins) on the target cell surface.[9] Herein, we report such a new example that a SPG-lactose conjugate can strongly bind asialoglycoprotein receptors on hepatocytes and mediate the following receptor-dependent endocytosis as well as effective transfection.

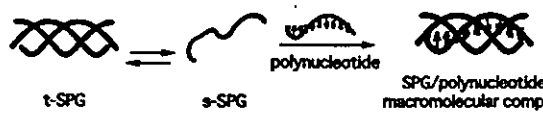

Fig. 1 Formation of the SPG/polynucleotide macromolecular complex.

SPG bearing lactose-appendages (SPG-Lac, Chart 1) were prepared through the following procedure.[10] Pendent glucosides of native SPG (MW 150 kDa) were selectively oxidized by treatment with aqueous $NaIO_4$ to afford an aldehyde-functionalized SPG, in which the β-1,3-glucan main-chain without the $NaIO_4$-sensitive 1,2-diol group remains intact. Schiff base formation between aldehyde-functionalized SPG and aminoethyl-lactoside was attained in DMSO and then, an excess amount of $NaBH_4$ was added to yield SPG tethering lactosides through secondary imino-linkages. We estimated the conversion ratio (n) based on the nitrogen contents by elemental analysis. SPG having a moderate content of lactose-appendages (n = 0.14) was thus obtained.

Macromolecular complexes composed of SPG-Lac and polynucleotides were easily prepared by mixing SPG-Lac in DMSO and polynucleotides in water. Proof of complex formation was obtained by CD spectra (Fig. 2(a)): The SPG-Lac/poly(dA) complex shows CD spectra in which a predominant negative peak (250 nm) observed for free poly(dA) was suppressed and new negative (265 nm) and positive (282 nm) peaks appeared. The observed CD spectral change is similar to that of the SPG/poly(dA) complex, indicating the formation of a hetero-triple-stranded macromolecular complex.[8] We measured the CD spectra at various temperatures (5–80 °C) to assess the thermal stability of the complex. The CD spectra of the SPG-Lac/poly(dA) complex are independent of the temperature up to 45 °C, at which point they suddenly changed with increasing temperature at around 50 °C into the CD spectra attributable to free poly(dA).[11] The finding proves

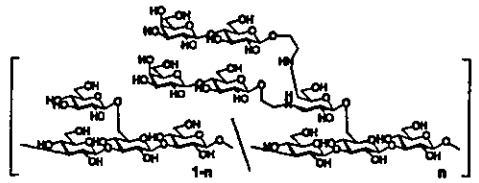

Chart 1 Structure of SPG carrying lactose appendages (SPG-Lac).

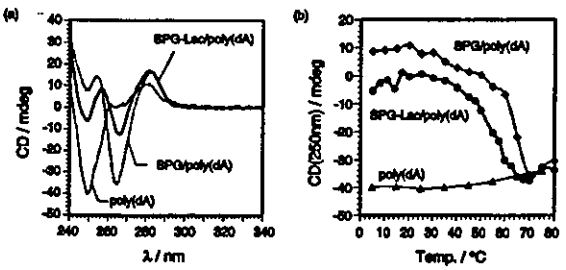

Fig. 2 (a) CD spectra of the SPG-Lac/poly(dA) complex, SPG/poly(dA) complex and poly(dA): [poly(dA)] = 0.08 mg ml^{-1}, [SPG-Lac] or [SPG] = 0.42 mg ml^{-1}, 5 °C, d = 1.0 cm, Tris-buffer (0.83 mM, pH 8.0). (b) Temperature dependence of the CD (250 nm) intensities.

† Electronic supplementary information (ESI) available: Procedures for the synthesis of aminoethyl-lactoside and a confocal fluorescent microscopic image. See http://www.rsc.org/suppdata/cc/b3/b313426a/
‡ Polysaccharide–Polynucleotide complexes, Part 26.

DOI: 10.1039/b313426a

that the complex is dissociated cooperatively at this temperature. The SPG-Lac/poly(dA) complex showed a melting temperature (T_m) at around 52 °C (Fig. 2(b)), which is lower than that (63 °C) of the SPG/poly(dA) complex but still sufficiently higher than physiological temperature.

The emission intensity of fluorescein isothiocyanate-labeled ricinus communis agglutinin (FITC-RCA_{120}, lactose-specific) was suppressed in the presence of the SPG-Lac/poly(dA) complex, suggesting their binding (Fig. 3(a)).[12] Together with the fact that neither poly(dA) nor the SPG/poly(dA) complex induces such fluorescence suppression, we can conclude that specific interactions occur between the complex and FITC-RCA_{120}.[13] Fig. 3(b) shows the relative fluorescence intensities (I/I_0) of FITC-RCA_{120} plotted against various concentrations of the macromolecular complexes. We estimated the RCA_{120} affinity ($K_{1/2}$) of the SPG-Lac/poly(dA) complex to be 14 μg ml^{-1} by using computational curve fitting.[14] The $K_{1/2}$ value based on [lactose-unit] is 1.7×10^{-6} M which is 120 times higher than that of monomeric lactose (2.1×10^{-4} M) owing to the clustering effect, and comparable to that of multi-lactosylated polyacrylamides (9.1×10^{-7} M).[12] These data clearly show that the SPG-Lac/poly(dA) complex can be strongly recognized by lactose-binding lectins and therefore can act as a hepatocyte-specific antisense carrier.

Specific lectin-binding of the SPG-Lac/poly(dA) complex was also demonstrated by confocal microscope observation using lectin-labeled agarose beads (~50 μm) and rhodamin-labeled $(dA)_{45}$. RCA_{120}-agarose was stained by the SPG-Lac/Rho-$(dA)_{45}$ complex (Fig. S1, ESI†), whereas agarose bearing ConA (α-Man/Glc specific) was not. These data also reveal that carrier/polynucleotide complexes are *not* dissociated upon binding to lectins and therefore, one can expect that the complexed polynucleotides are up-taken by the target cell *via* receptor-mediated endocytosis without unfavourable release at the cell surface.

Receptor-mediated antisense transfection by SPG-Lac was demonstrated using human hepatocytes (HepG2) and $(dA)_{40}$-tagged phosphorothioate-type antisense (AS-c-myb: 5′-GTGCCGGGGTCTTCGGGC-$(A)_{40}$-3′) which blocks the proto-oncogene mRNA translation.[15] HepG2 cells were cultured in the presence of the SPG-Lac/As-c-myb complex, SPG/As-c-myb complex or free As-c-myb ([As-c-myb] = 30 μg ml^{-1}) and then, the cell-numbers were counted by using the Cell Counting Kit-8 (Dojin).[16] The SPG-Lac/As-c-myb complex was found to suppress the cell-growth effectively (52 ± 4%) in comparison to the SPG/As-c-myb complex (81 ± 6%) and free As-c-myb (91 ± 6%). Together with the facts that HepG2 expresses lactose-receptors on the cell surface and that monomeric lactose (20 mM) reduces the transfection effect (79 ± 5%), specific lactose-protein interactions and the following receptor-mediated endocytosis should be responsible for the enhanced transfection effect observed for the SPG-Lac/As-c-myb complex.

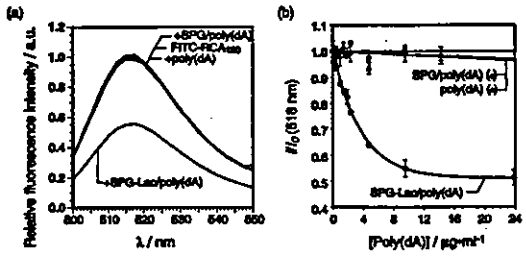

Fig. 3 (a) Fluorescence spectra of FITC-RCA_{120} in the presence of free poly(dA) (9 μg ml^{-1}) and that complexed with SPG derivatives (46 μg ml^{-1}): Tris-buffer (20 mM, pH 7.2), 25 °C, λ_{ex} = 490 nm. (b) Relative fluorescence intensities (I/I_0) of FITC-RCA_{120} at various concentrations of the SPG-Lac/poly(dA) complex, SPG/poly(dA) complex and free poly(dA).

In conclusion, a SPG-derivative carrying lactose-appendages was prepared *via* oxidation followed by reductive amination. Easy preparation, high stability, excellent lectin-affinity and effective antisense-transfection ensure the potential of SPG-lactose conjugates as new hepatocyte-targeted antisense carriers.

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Notes and references

1 T. V. Chirila, P. E. Rakoczy, K. L. Garrett, X. Lou and I. J. Constable, *Biomaterials*, 2002, **23**, 321; I. Jääskeläinen, S. Peltola, P. Honkakoshi, J. Mönkkönen and A. Urtti, *Eur. J. Pharm. Sci.*, 2000, **10**, 187.

2 K. Kataoka, H. Togawa, A. Harada, K. Yasugi, T. Matsumoto and S. Katayose, *Macromolecules*, 1996, **29**, 8556.

3 O. Boussif, F. Lezoualch, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and P. Behr, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 7297.

4 J. F. Kukowska-Latallo, A. U. Bielinska, J. Johnson, R. Spindler, D. A. Tomalia and J. R. Baker, Jr., *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 4897.

5 A. Bot, H. E. Smorenburg, R. Vreeker, M. Paques and A. H. Clark, *Carbohydrate Polym.*, 2001, **45**, 363; H. Saito, Y. Yoshioka, N. Uehara, J. Aketagawa, S. Tanaka and Y. Shibata, *Carbohydrate Res.*, 1991, **217**, 181.

6 T. Yanaki, T. Norisuye and H. Fujita, *Macromolecules*, 1980, **13**, 1462; T. Norisuye, T. Yanaki and H. Fujita, *J. Polym. Sci. Polym. Phys. Ed.*, 1980, **18**, 547; C. T. Chuah, A. Sarko, Y. deslandes and R. H. Marchessault, *Macromolecules*, 1983, **16**, 1375.

7 K. Sakurai and S. Shinkai, *J. Am. Chem. Soc.*, 2000, **122**, 4520; K. Sakurai, M. Mizu and S. Shinkai, *Biomacromolecules*, 2001, **2**, 641; K. Koumoto, T. Kimura, K. Sakurai and S. Shinkai, *Bioorg. Chem.*, 2001, **29**, 178.

8 K. Koumoto, M. Mizu, K. Sakurai, T. Kunitake and S. Shinkai, *Chem. Diversity*, in press; M. Mizu, K. Koumoto, T. Kimura, K. Sakurai and S. Shinkai, *Biomaterials*, in press.

9 D. T. Klink, M. C. Glick and T. F. Scanlin, *Glycoconjugate J.*, 2002, **18**, 731; Y. Koyama, E. Yamada, T. Ito, Y. Mizutani and T. Yamaoka, *Macromol. Biosci.*, 2002, **2**, 251.

10 Aminoethyl-lactoside was synthesized from *per*-acetyllactose *via* glycosylation with 2-bromoethanol, azidation and hydrogenation followed by deacetylation. See ESI† for detailed procedures and characterization.

11 M. Mizu, T. Kimura, K. Koumoto, K. Sakurai and S. Shinkai, *Chem. Commun.*, 2001, 429.

12 T. Hasegawa, S. Kondoh, K. Matsuura and K. Kobayashi, *Macromolecules*, 1999, **32**, 6595.

13 We also confirmed that the addition of SPG-Lac/poly(dA) does not induce any change in the fluorescence spectrum of FITC-ConA.

14 The formation of a 1:1 complex is assumed and computational curve fitting was carried out by using the following equation, $I/I_0 = 1 - A\{1 - \exp(-[\text{SPG-Lac}]/K_{1/2})\}$, where A and $K_{1/2}$ are the maximum extent of emission quenching and the concentration of SPG-Lac to induce half-maximum quenching, respectively.

15 A. M. Gewirtz and B. Calabretta, *Science*, 1988, **242**, 1303; M. Mizu, K. Koumoto, T. Kimura, K. Sakurai, T. Nagasaki and S. Shinkai, *Bull. Chem. Soc. Jpn.*, submitted.

16 The HepG2 cell line was cultured at 37 °C in MEM (Gibco) supplemented with non-essential amino acids (0.1 mM), sodium pyruvate (10 μM), FBS (10%) and penicillin-streptomycin mixture (1 wt%). Cells were grown in a humidified air containing 5% CO_2.

Curdlan and Schizophyllan (β-1,3-Glucans) can Entrap Single-wall Carbon Nanotubes in Their Helical Superstructure

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We have found that s-SPG and curdlan are capable of wrapping SWNTs and the "periodical" helical structure is created, reflecting the helical nature of the SPG main-chain, on the SWNTs surface. The novel SWNTs/β-1,3-glucan composites were characterized by TEM and AFM, which consistently support the view that these β-1,3-glucans wrap SWNTs, creating a helical higher-order structure.

Since discovery of single-wall carbon nanotubes (SWNTs),[1] they have been expected to become a new potential source of functional materials.[2] However, their strong cohesive nature and poor solubility have caused the researchers trouble for a long time, because these properties seriously hamper for the researchers to obtain the reproducible data. One potential solution to overcome these problems is to wrap SWNTs by polymers. The polymers that are known to have such a wrapping ability are poly(vinylpyrrolidone),[3] poly(styrenesulfonate),[3] poly(metaphenylenevinylene),[4] amylose,[5] DNA,[6] peptides,[7] etc. Among them, particularly interesting is amylose, because (1) it has almost no light absorption in UV–vis wavelength region, so that the dispersed composites are very suitable for photochemical experiments, (2) the sugar-coated surface of the composite should show the bio-compatibility which makes their medicinal applications possible, and (3) if the sugar group is recognized by proteins (such as lectins), one may construct novel SWNTs-based supramolecular network structures.[8]

Schizophyllan (SPG) is a natural polysaccharide produced by fungus *Schizophyllum commune* and its repeating unit consists of three β-(1–3) glucoses and one β-(1–6) glucose side chain linked at every third main-chain glucose (Figure 1).[9] SPG adopts a triple helix (t-SPG) in nature, which can be dissociated into a single chain (s-SPG) by dissolving in dimethyl sulfoxide (DMSO).[10] The s-SPG chain can retrieve the original triple helix by exchanging DMSO for water.[11] Recently, we found its novel solution property that when this renaturing process is carried out in the presence of polynucleotides, the resultant triple helix consists of two s-SPG chains and one nucleotide chain.[12] Taking the facts into consideration that (1) the diameter of t-SPG (2.6 nm) is comparable with that of SWNTs (1–2 nm), (2) DNAs, which are complexed by s-SPG, are used as a SWNTs solubilizer,[6] and (3) η-cyclodextrin is capable of forming a pseudo-rotaxane-type complex with SWNTs,[13] it is very likely that s-SPG wraps SWNTs, forming a "periodical" helical structure. To the best of our knowledge, there is no direct evidence for the creation of the regular structure from SWNTs and water-soluble polymers.[14] We here report that s-SPG and curdlan (β-1,3-glucan without a side-chain glucose) are capable of wrapping SWNTs and the "periodical" helical structure is created, reflecting the helical nature of the SPG main-chain, on the SWNTs surface.

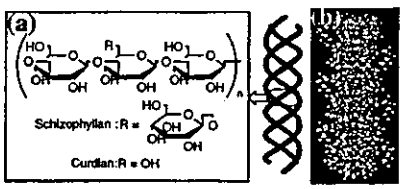

Figure 1. (a) Repeating unit of Schizophyllan and Curdlan; (b) representative model of the Schizophyllan triplehelix.

SWNTs were cut into the appropriate length as described previously.[15] The length as estimated by AFM was between 1 and 3 μm. An aqueous solution of dispered SWNTs was mixed with a DMSO solution containing polysaccharide (Mw = 150000 for s-SPG, 33000 for curdlan, and 15000 for amylose).[16] At this stage, the water/DMSO mixed solution contained 300–600 μg mL^{-1} of SWNTs and 3.3 mg mL^{-1} of polysaccharide. After leaving it at 50 °C for 2 days, the mixture was treated with a centrifuge (7000 rpm) for 1 h and the supernatant which contains the unreacted s-SPG was pipetted off. The precipitated SWNTs-polysaccharide complexes were then dispersed into water (200 μL). Repeating this process, excess amount of s-SPG was removed and the solvent was replaced by water. To find an optimum mixed solvent we prepared the samples, changing the composition of water/DMSO (v/v) in the range from 50/50 to 92/8. The concentration of the solubilized SWNTs was estimated with the absorbance at 500 nm in UV–vis spectroscopy.[17] We found that the highest solubility is attained at water/DMSO = 84/16 (v/v): the percentages of solubilized SWNTs are 85.5, 69.5, 73.5% of the feed in the presence of s-SPG, curdlan, and amylose, respectively. Hereafter, we employed this mixed solvent as a standard medium. In the absence of polysaccharide, on the other hand, SWNTs were scarcely dissolved under the same treatment.

TEM images of SWNTs themselves and their composites with s-SPG are shown in Figure 2. Figure 2a shows that the length of SWNTs is several μm, which is consistent with the length estimated by AFM. However, the diameter size is somewhat larger, indicating that they are easily bundled in the absence of solubilizers. In the presence of s-SPG or curdlan, on the other hand, SWNTs still exist as thin fibers, suggesting that the wrapping by polysaccharides suppresses the aggregation of SWNTs (Figures 2b and 2c). To obtain a clearer image of the composite, we magnified one fibril after staining with phosphotungstic acid. Very interestingly, this fibril consists of a few bundles with ca. 8-nm diameter and each bundle is covered by oblique stripes (Figure 2d). This pattern shows that s-SPG twines round a SWNT (or a few SWNTs) according

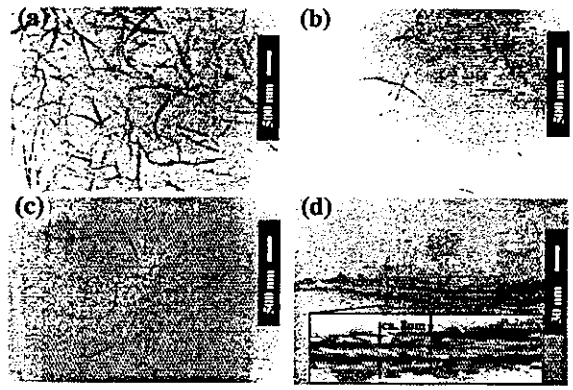

Figure 2. TEM images of (a) SWNTs; (b) SWNTs/s-SPG composite; (c) SWNTs/curdlan composite; (d) magnified picture of (b), (a) -(c) were taken without staining whereas (d) was taken after staining with 2.0 wt% phosphotungstic acid.

to a periodical helical motif. As reference experiments, we prepared the samples of SWNTs and s-SPG separately according to the same treatment. However, we could not recognize the oblique stripes at all.

To obtain unequivocal evidence that these twined polysaccharides really constitute a periodical helical structure around SWNTs, we observed these fibrils by AFM. As shown in Figure 3a, the surface of SWNTs themselves did not give any specific pattern. We also prepared SWNTs solutions dispersed by amylose or poly(vinylpyrrolidone), but their AFM images are similar to that in Figure 3a. This implies that even though these water-soluble polymers can wrap SWNTs to dissolve them into water, the regular, periodical structure is not constructed in their wrapping process. On the other hand, when a DMSO solution of s-SPG or curdlan was cast on mica, we could observe a fine polymeric network structure (data not shown here). Very interestingly, when SWNTs were dispersed with the aid of s-SPG or curdlan into water, the surface of the fibrous aggregates gave an oblique stripe structure (Figures 3b–3d), similar to that observed by TEM. As reference experiments, we prepared the samples of SWNTs and t-SPG according to the same treatment. However, we could not recognize the oblique stripes at all. This result shows the fact that the renaturing process is essential for the composite formation.

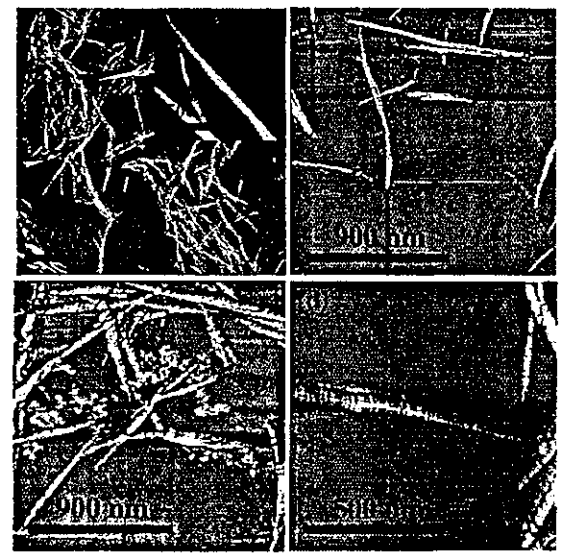

Figure 3. AFM images of (a) SWNTs;(b)SWNTs/s-SPG composite;(c) SWNTs/curdlan composite;(d) SWNTs/s-SPG composite (magnified picture).

To obtain further evidence that these fibers are composites of SWNTs and s-SPG (or curdlan), we characterized them by spectroscopic methods. Firstly, the presence of SWNTs was evidenced by vis–NIR spectrum. The characteristic absorption band is seen around 500–800 nm and 1200–1800 nm regions, which are basically similar to those of reported spectra.[18] It is known that SWNTs have characteristic Raman peaks at around 190 and 1590 cm^{-1}.[19] We have also confirmed that SWNTs/s-SPG and SWNTs/curdlan composites give the peaks at 188 and 1591 cm^{-1}.

As a summary of the foregoing findings, one can now conclude that the nanorods observable with the microscopes are composites formed from SWNTs and s-SPG (or curdlan). To the best our knowledge, this is the first time that the helical twining of SWNTs by water-soluble polymers are visually confirmed. We believe that the clear images obtained here are due to the inherent property of β-1,3-glucans to form a helical higher-order structure. Further chemical and physical characterizations and medicinal applications are currently continued.

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References and Notes

1 S. Iijima, *Nature*, 354, 56 (1991).
2 R. H. Baughman, A. A. Zakhidov, and W. A. de Heer, *Science*, 297, 787 (2002).
3 M. J. O'Connell, P. Boul, L. M. Ericson, C. Huffman, Y. Wan, E. Haroz, C. Kuper, J. Tour, K. D. Ausman, and R. E. Smalley, *Chem. Phys. Lett.*, 342, 265 (2001).
4 A. Star, J. F. Stoddart, D. Steuerman, M. Diehl, A. Boukai, E. W. Wong, X. Yang, S.-W. Chung, H. Choi, and J. R. Heath, *Angew. Chem., Int. Ed.*, 40, 1721 (2001).
5 a) A. Star, D. W. Steuerman, J. R. Heath, and J. F. Stoddart, *Angew. Chem., Int. Ed.*, 41, 2508 (2002). b) O.-K. Kim, J. Je, J. W. Baldwin, S. Kooi, P. E. Phehrsson, and L. J. Buckley, *J. Am. Chem. Soc.*, 125, 4426 (2003).
6 a) M. Zheng, A. Jagota, E. D. Semke, B. A. Diner, R. S. Mclean, S. R. Lustig, and R. E. R. Tassi, *Nat. Mater.*, 2, 338 (2003). b) N. Nakashima, S. Okuzono, H. Murakami, T. Nakai, and K. Yoshikawa, *Chem. Lett.*, 32, 456 (2003).
7 D. Pantarotto, C. D. Partidos, R. Graff, J. Hoebeke, J.-P. Briand, M. Prato, and A. Bianco, *J. Am. Chem. Soc.*, 125, 6160 (2003).
8 A supramolecular network was made from sugar-modified SWNTs and lectin: K. Matsuura, K. Hayashi, and N. Kimizuka, *Chem. Lett.*, 32, 212 (2003).
9 a) K. Yanaki, W. Ito, T. Kojima, T. Norisuye, N. Takano, and H. Fujita, *Biophys. Chem.*, 17, 337 (1983). b) K. Tabata, W. Ito, T. Kojima, S. Kawabata, and A. Misaki, *Carbohydr. Res.*, 89, 121 (1981).
10 T. Yanaki, T. Norisuye, and M, Fujita, *Macromolecules*, 13, 1462 (1980).
11 T. M. McIntire and D. A. Brant, *J. Am. Chem. Soc.*, 120, 6909 (1998).
12 K. Sakurai and S. Shinkai, *J. Am. Chem. Soc.*, 122, 4520 (2000).
13 H. Dodziuk, A. Ejchart, W. Anczewski, H. Ueda, E. Krinichnaya, G. Dolgonos, and W. Kutner, *Chem. Commun.*, 2003, 986.
14 Kim et al. reported that amylose-coated SWNTs give twisted structures about 30-nm diameter (Ref 5b). Taking its large diameter and loosely twisted SEM and AFM images into consideration, the observed twisted fibrils would not be the amylose polymer chain but the aggregated bundles of amylose-SWNT composites. In the present system, on the other hand, we can confirm that the helical structures are constructed by β-1,3-glucan polymer chain.
15 M. Sano, A. Kamino, J. Okamura, and S. Shinkai, *Science*, 293, 1299 (2001).
16 Native curdlan (M_w = 1200000) is scarcely soluble in water, but when the M_w is reduced, it becomes moderately water-soluble: K. Koumoto, T. Kimura, H. Kobayashi, K. Sakurai, and S. Shinkai, *Chem. Lett.*, 2001, 908.
17 J. L. Bahr, E. T. Mickelson, M. J. Bronikowski, and R. E. Smalley, J. M. Tour, *Chem. Commun.*, 2001, 193.
18 I. W. Chiang, B. E. Brinson, A. Y. Huang, P. A. Willis, M. J. Bronikowski, J. L. Margarve, R. E. Smalley, and R. H. Hauge, *J. Phys. Chem. B*, 105, 8297 (2001).
19 A. M. Rao, E. Richter, S. Bandow, B. Chase, P. C. Eklund, K. A. Williams, S. Fang, K. R. Subbaswamy, M. Menon, S. Thess, R. E. Smalley, and G. Dresselhaus, *Science*, 275, 187 (1997).

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Chemically modified polysaccharide schizophyllan for antisense oligonucleotides delivery to enhance the cellular uptake efficiency

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Abstract

Schizophyllan is a natural β-(1 → 3)-D-glucan existing as a triple helix in water and as a single chain in dimethylsulfoxide (DMSO), respectively. As we already reported, when some homo-phosphodiester polynucleotide (for example, poly(dA) or poly(C)) is added to the schizophyllan/DMSO solution and subsequently DMSO is exchanged for water, the single chain of schizophyllan forms a complex with the polynucleotide. Furthermore, we have already demonstrated that one of the potential applications of this novel complex is an antisense-oligonucleotide (AS ODN) carrier. This work describes a versatile and universal modification technique which enables us to introduce various functional groups only to the side chain of schizophyllan. This technique consists of periodate oxidation of the glucose side chain (it does not react with the main chain because of the absence of the 1,2-diol group in β-(1 → 3)-glucan) and subsequent introduction of the functional groups into the formyl terminate. In the present work, the introduced functional groups were spermine, octa-arginine (R8), arginine–glycine–aspartic acid tripeptide (RGD) and some amino or α-amino acid compounds. Using these compounds, we made the complexes and carried out an in vitro antisense assay for them, administrating a phosphorothioate AS ODN to the melanoma A375 or leukemia HL-60 cell lines to depress their *c-myb* mRNA. When we used the R8 or RGD modified schizophyllan as the antisense carrier, the antisense effect was most enhanced among others. Their superiority can be ascribed to enhancement of endocytosis due to these functional peptides. Furthermore, the cytotoxicity for these two modified schizophyllans was negligibly as small as the natural (unmodified) schizophyllan. One of the peculiar features of our system is that the complex (i.e., carrier + AS ODN) is charged negatively in total, which is different from the conventional systems. The present work has thus clarified that schizophyllan can act as a new potential candidate for AS ODN carriers.

Keywords: Antisense-oligonucleotide carrier; Schizophyllan; Polysaccharide–polynucleotide complex; β-1,3-glucan

1. Introduction

Antisense oligonucleotides (AS ODNs) are applied extensively to silencing single genes exploiting their exquisite specificity to match the target mRNA [1–3]. The principles and mechanisms of the antisense therapy are (1) to deliver AS ODNs to the cells, (2) to induce the cellular uptake of AS ODNs, (3) to release AS ODNs to cytosol, (4) to bind to a particular mRNA to make a DNA/RNA duplex, and (5) to make RNase H cleave the duplex so as to inhibit the protein expression. The major advantage of this strategy over the conventional drugs is related to the potential specificity of the action. Theoretically, an oligonucleotide can be designed

Abbreviations: AS ODN, antisense oligonucleotide; S ODN, sense oligonucleotide; AS-c-myb, 5′-GTGCCGGGGTCTTCGGGC-$(dA)_{40}$-3′ phosphorothioate, an AS ODN for *c-myb* mRNA; S-c-myb, 5′-CACGGCCCCAGAAGCCCG-$(dA)_{40}$-3′ phosphorothioate, S ODN for *c-myb* mRNA; s-SPG, single chain of schizophyllan; s-SPG/AS ODN or modified s-SPG/AS ODN, complexes made from s-SPG and AS ODN, or modified s-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 necessarily 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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to target any single gene in the human genome. Therefore, inhibition in 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; instability of AS ODNs in biological fluids and low uptake efficiency into the target cells [1,3]. The instability of AS ODNs is mainly ascribed to two factors; the hydrolysis mediated by deoxyribonuclease and non-specific binding to proteins. The later event can induce a "non-antisense effect", when oligonucleotide analogues such as phosphorothioates is used [4]. However, the hydrolysis can be overcome by use of oligonucleotide analogues such as phosphorothioates, phosphoramidates, and peptide nucleic acids [5]. Especially, phosphorothioates are the leading candidates among the first generation of antisense compounds and several of them are indeed in phase I/II clinical trial [6,7].

When we use phosphorothioates as AS ODNs, non-specific binding to some proteins is the major obstruction [4]. It is reasonably considered that the materials which can form a complex with phosphorothioates AS ODNs can be used as a carrier. Several materials are studied as AS ODN carriers [1,3,8–10,12–18]. Cationic lipids can form a complex with AS ODNs and encapsulate them in the liposome [1,3,8–10]. 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 [11], leading to a short lifetime in the serum. Furthermore, the size of the liposome is usually too large for cells [3]. Synthetic polycations, such as poly(L-lysine) [9] and polyethyleneimine (PEI) [12,13] have been studied as an AS ODN carrier, because polycations can form polyion complexes with polynucleotides. Although polycations have great advantage to improve the cellular uptake, serious drawbacks are pointed out, such as toxicity of the polycations and poor solubility of the resultant polyion complexes [3]. Recently, various ideas are proposed to improve these issues [14–17], and additionally, some of them can provide a targeting or intellectual-release device. However, the cytotoxicity due to artificially synthesized materials still remains as a serious problem. Utilizing natural polysaccharides is considered to be a good and new approach to design the AS ODN carrier [18]. This is because natural polysaccharides can biodegrade into non-toxic components and can provide satisfactory solubility. However, no one had ever thought that polysaccharides themselves could be used as an AS ODN binding portion, until Sakurai and Shinkai [19,20] found for the first time that schizophyllan can form a complex with some polynucleotides.

Schizophyllan 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 links to the main chain at every three glucose residues (see Fig. 1 for the chemical structure)

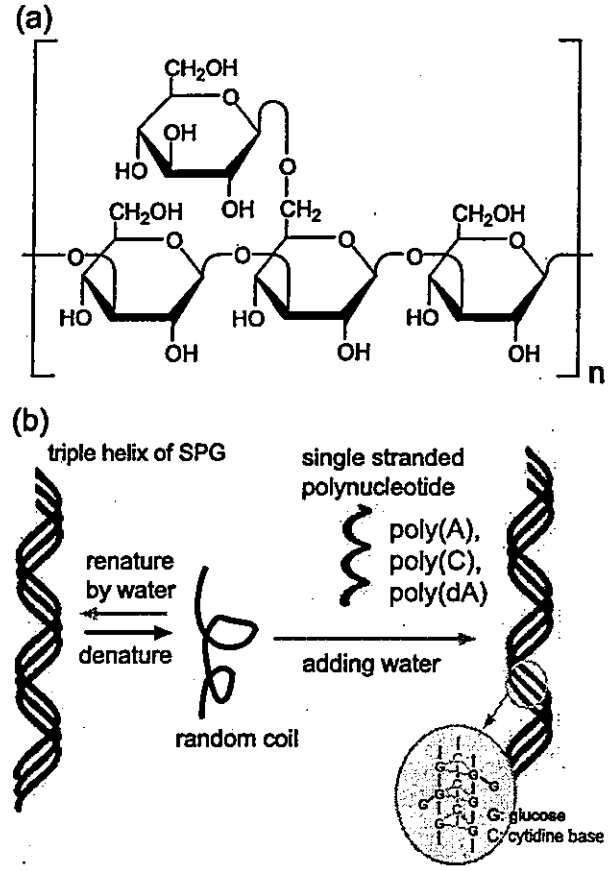


Fig. 1. Repeating units of schizophyllan (a) and a schematic illustration of the complexation (b). In (b), the blue and red lines represent the s-SPG and polynucleotide molecules, respectively. The inset shows the proposed stoichiometric model for the complex; the capital letters of G and C represent the glucose and base, and the blue lines show the hydrogen bonds, respectively.

[21]. Schizophyllan adopts a triple-helical conformation in water and a random coil in dimethylsulfoxide (DMSO) [22,23]. 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 [24,25]. Recently, Sakurai and Shinkai [19,20] found that 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 (see Fig. 1(b)). Their data showed that the 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 the same complex as schizophyllan; therefore, this complexation is generally more commonly observed in nature for β-(1 → 3)-D-glucans [26,27]. Some of other novel features for this complex are (1) the complex is remarkably stable (large binding constant) and considerably water-soluble in the physiological conditions [19,20], (2) the complexation

occurs in a highly stoichiometrical manner and the stoichiometric number indicates that two schizophyllan units and three base units are interacting with each other ((see Fig. 1(b)) [20], (3) when a s-SPG/DNA complex meets the corresponding complementary sequence, for example s-SPG/poly(T) meets poly(dA), the complex is dissociated immediately and the hybridization takes place [28]. When schizophyllan is used as an AS ODN carrier, the three features of (1)–(3) mentioned above seem greatly advantageous. In this paper, we make attempts to modify s-SPG to enhance the cellular uptake and carry out an in vitro assay to evaluate the ability as AS ODN carriers.

2. Strategy and chemistry

2.1. Strategy to deliver AS ODNs to cells by protecting them

Fig. 2 schematically presents our strategy to deliver AS ODNs to cells. Schizophyllan has no ability to bind to the plasma membrane to induce endocytosis or other uptake processes (see Fig. 10) [29]. Furthermore, it is known that administration of schizophyllan hardly elicits the immune response [30], so that schizophyllan may not be recognized by T cell receptors. Therefore, we need to modify the schizophyllan chain with a functional group which can induce the uptake of AS ODNs. Such functional groups can be cationic groups [1,3], a specific peptide which can bind to an integrin receptor on the plasma membrane [31], or other peptides corresponding to the human immunodeficiency virus or its analogs [32,33]. It should be emphasized that the modification has to be done only for the side chain of schizophyllan, because the β-(1 → 3)-D-glucan main chain is essentially important to form the complex. This site-specific modification can be successfully carried out, by oxidizing schizophyllan with sodium periodate anion to yield the formyl terminate [34], and subsequent reactions to attach a functional group to the formyl group. The resultant modified s-SPG and an AS ODN sequence can form a complex, which can be administrated in vivo or vitro (in this paper, only in vitro assay was carried out). Since we already know that the complex can protect the bound AS ODNs against nuclease-mediated hydrolysis or unfavorable binding to the serum proteins [35], the survival of the AS ODN population before reaching the cells should be increased. Once the complex has reached the cell, the binding site starts to interact with the cell surface to induce the cellular uptake.

The mechanism for the cellular uptake of AS ODNs differs from the introduced functional groups. Generally, the cellular membrane is negatively charged, so that cations can

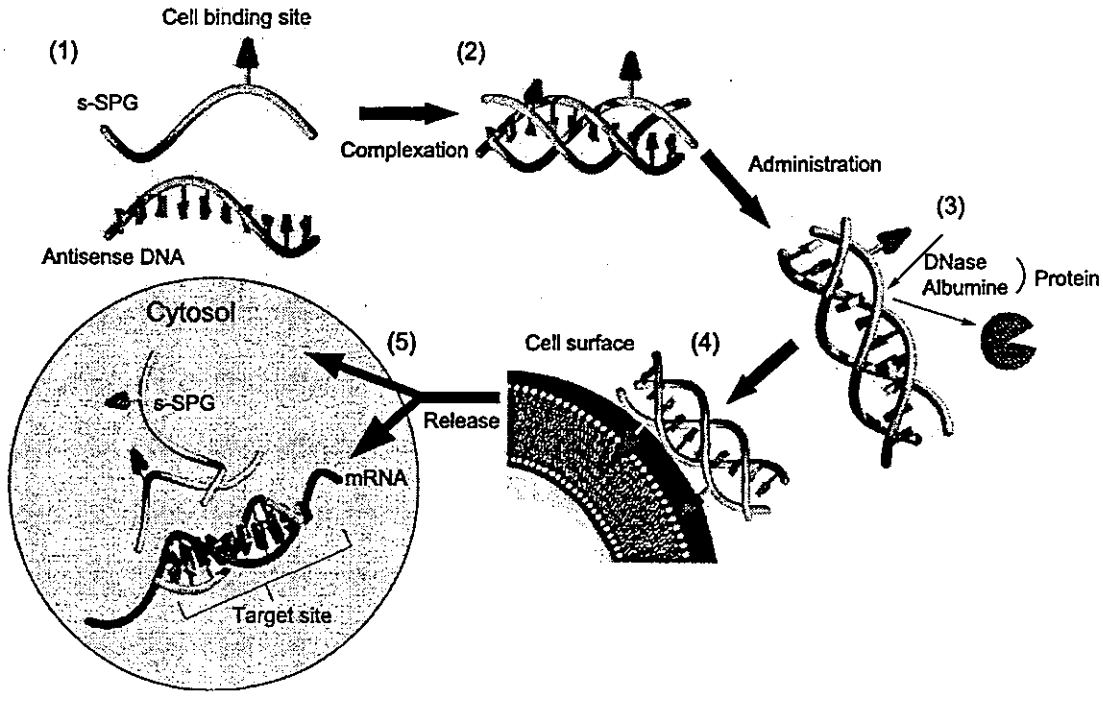


Fig. 2. The schematic illustration of our strategy to deliver AS ODNs to cells. As shown in (1), a functional group which can induce the cellular uptake of the complex is attached to the s-SPG side chain, where s-SPG stands for the single chain of schizophyllan. When the modified s-SPG and AS ODN is mixed, they form a complex consisting of two modified s-SPG and one AS ODN chains as presented in (2) [20]. As clarified in Refs. [29,35], the complex can prevent the bound AS ODN from forming unfavorable interactions with proteins (3). As shown in (4), once the functional group binds to the plasma membrane, the cellular uptake can be induced to ingest the complex. As clarified in Ref. [28], when the s-SPG/AS ODN complex meets the corresponding complementary sequence of mRNA, the complex dissociates immediately to release the AS ODN and hybridization takes place.

bind to the surface with the Coulombic forces. Since there should be no specific receptor for cations on the membrane, the bound cations or cation-attached complexes should be ingested by the regular pinocytosis cycle. As mentioned in the Introduction, many studies have employed cationic lipids or polycations to deliver AS ODNs. In these cases, cationic carriers must contain enough charge to neutralize the AS ODN and also to provide the sufficient residual charge for interaction with the cell membrane. Such a large amount of cations can cause serious cytotoxicity. Furthermore, it is sometimes difficult to release the bound AS ODN from the ion complex due to the formation of stable complexes such as PEI/AS ODN complexes. In the s-SPG/AS ODN complex, the AS ODN is bound to s-SPG with the hydrogen bonding interaction, so that we do not need a large amount of cations. It should be pointed out that the feature of the present system is completely different from other carriers.

As a peptide which can bind to an integrin receptor, we attached an arginine–glycine–aspartic acid tripeptide (RGD). Integrins are natural targets for receptor-mediated cell entry used by a variety of bacteria and viruses [36,37], so that when RGD is attached to liposomes, the increment of the cellular uptake is already demonstrated [31]. Since RGD is bound to integrins, the cellular uptake is considered to be achieved through a receptor-mediated endocytosis. Therefore, the AS ODNs have to survive through the hydrolytic-enzyme rich and acidic atmosphere in lysosomes. Since we already know that the complex dissociates in the acidic solutions (pH < 5–6) [38], phosphorothioates AS ODNs should be better than phosphodiester AS ODNs in our study. In contrast to RGD, the arginine-rich peptides corresponding to the human immunodeficiency virus, such as HIV-1 Rev-(34–50) or HIV-1 Tat-(48–60), induce a cellular uptake by the different pathway from receptor-mediated endocytosis [33]. Suzuki et al. [33] demonstrated that even octa-arginine (R8) induces the same effect as HIV-1 Rev-(34–50) or HIV-1 Tat-(48–60). In our study, we attached R8 to s-SPG. Although the detailed mechanism is not clarified, the pathway induced by R8 is called 'potocytosis' [39].

2.2. Side chain-specific chemical modification

It is essentially important to introduce the abovementioned functional groups 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 or AS ODNs [26,27], thus it is better to keep the main chain intact during the reaction [34,40]. We already established such a fine chemical technique [34,40], which is presented in Fig. 3(I). The key step is oxidation of the side chain glucose by periodate anion (IO_4^-), which leads to cleavage of the pyranose ring and converts each end to the formyl terminate. When we attached 2-aminoethanol, spermine, *N*,*N*′-bis(3-aminopropyl)-1,3-propanediamine, Arg and Ser, the carboxyl group reacted with the amine or amino acids (ii in the figure) and the subsequent reduction (iii) of the resultant Schiff base produced the final products. According to our previous work [34,40], we confirmed that the molecular weight was not changed through these reaction processes, and the side chain-modified s-SPG can form a complex, and the stability of the complex is increased because the ion-pair formation between the phosphate anion and the introduced cation. It should be pointed out that about 10 mol% of the modification does not induce any spectral changes in circular dichroism, indicating that the complex structure is not altered by the modification [34]. When we introduced the peptides to the side chain, we applied a conventional method with the addition of cysteine to the unsaturated bond in the maleimide moiety (v and vi in Fig. 3).

All modified s-SPG derivatives were characterized by nitrogen elemental analysis to determine the modification

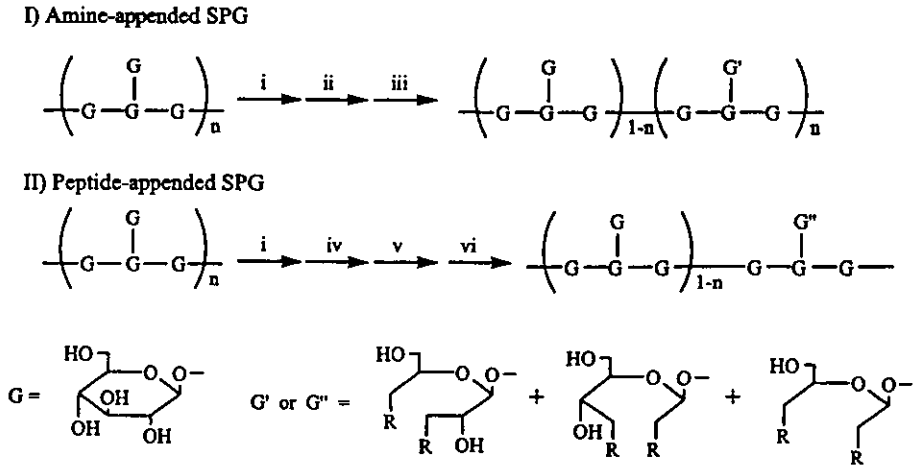
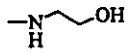
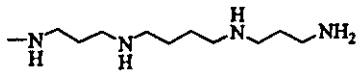
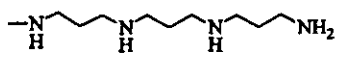
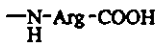
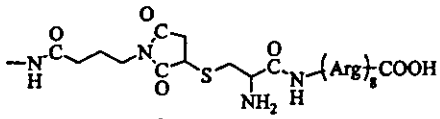
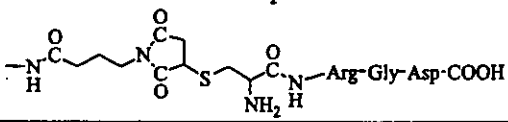


Fig. 3. Reaction scheme to introduce the functional groups into the side chain of s-SPG.

Table 1
Sample codes and the introduced chemical groups

Sample code	R	Modification level[a]	N/P ratio[b]
N(17)-SPG		17.1 ±0.7 mol%	0.51
SP(4.6)-SPG		4.6 ±0.3 mol%	0.27
SP(12.7)-SPG		12.7 ±0.9 mol%	0.77
SP(24.7)-SPG		24.7 ±1.3 mol%	1.50
APPD(4.1)-SPG		4.1 ±0.3 mol%	0.26
R(3.6)-SPG		3.6 ±0.1 mol%	0.21
R(9.3)-SPG		9.3 ±0.2 mol%	0.56
R(13.5)-SPG		13.5 ±0.1 mol%	0.81
S(6.4)-SPG		6.4 ±0.3 mol%	-0.20
S(13.2)-SPG		13.2 ±0.4 mol%	-0.39
R8(0.5)-SPG		0.5 ±0.1 mol%	~0
RGD(1.3)-SPG		1.3 ±0.3 mol%	~0

[a] Determined by N elemental analysis.
[b] Cation(N)/anion(P) ratio.

level. The samples and their codes are presented in Table 1 as well as the introduced functional groups.

3. Results and discussion

3.1. Complexation between phosphorothioate AS ODNs and schizophyllan

The sequence of 5′-GTGCCGGGGTCTTCGGGC-3′ is well-known to bind to *c-myb* mRNA and to lead drastic depression of *c-myb* [41–46] in many cell lines. Here, *c-myb* is a proto-oncogene encoding a nuclear transcription factor, and its over-expression usually leads to cancer. As our previous work revealed [35,47], short and hetero-ODNs cannot bind to s-SPG, so that we had to attach a poly(dA)$_{40}$ tail (which has 40 dA bases) at the 3′ end 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, hereinafter. By the way, our previous studies [29] have already shown that when s-SPG is not chemically modified, it can bind to AS-c-myb. The complex is stable at the cell incubation (37 °C) temperature. Since all modifications were applied to only the side chain, the modified s-SPG samples are expected to bind to AS-c-myb. We reconfirmed this expectation by gel electrophoresis.

Fig. 4 presents the gel electrophoresis migration patterns for mixtures of AS-c-myb and the modified s-SPG, comparing with naked AS-c-myb and a mixture of AS-c-myb and s-SPG. Here, only AS-c-myb is stained by GelStar®. AS-c-myb itself migrates, however, the mixtures of AS-c-myb and the modified s-SPG do not migrate at all, except for AS-c-myb + R(13.5)-SPG and AS-c-myb + S(13.2)-SPG. It has been demonstrated that once the complex is formed, it does not migrate at all in the agarose gel [20,29]. Therefore, the results in the figure indicate that the complex is formed in the mixture of AS-c-myb and the modified s-SPG, except for the R(13.5)-SPG and S(13.2)-SPG systems. We found that R(13.5)-SPG and S(13.2)-SPG were not completely soluble in water, providing some turbidity when we mixed the AS-c-myb solution with the R(13.5)-SPG or S(13.2)-SPG/DMSO solution. Therefore, it is presumed that all of the AS-c-myb molecules were not incorporated into the complex. Furthermore, the Ser residue in S(13.2)-SPG carries an anionic charge at pH = 8, so that the anion–anion repulsion between Ser and AS-c-myb may be another reason for the presence of the free AS-c-myb.

3.2. Enhanced antisense effect by the chemical modification, assayed with the melanoma cell lines; A375

Fig. 5 shows the A375 cell growth after cultured for 3 days, comparing all combinations of AS-c-myb and every carrier, including Lipofectin and unmodified s-SPG systems. Although not all data are included, the numerical results are also summarized at the left side of the histograms. In Fig. 5, the AS-c-myb doses were 12.5, 25 and 50 μg/ml (unfilled bar, hatched bar and filled bar, respectively). The cell growth is defined by the number of cells normalized by that of the control (without any addition of AS-c-myb nor s-SPG). In all cases, the s-SPG/AS-c-myb molar ratio is fixed at 1.5:1.0 because the previous work showed that the cell growth reaches the minimum around this ratio [29]. As mentioned in the Introduction, the complex consists

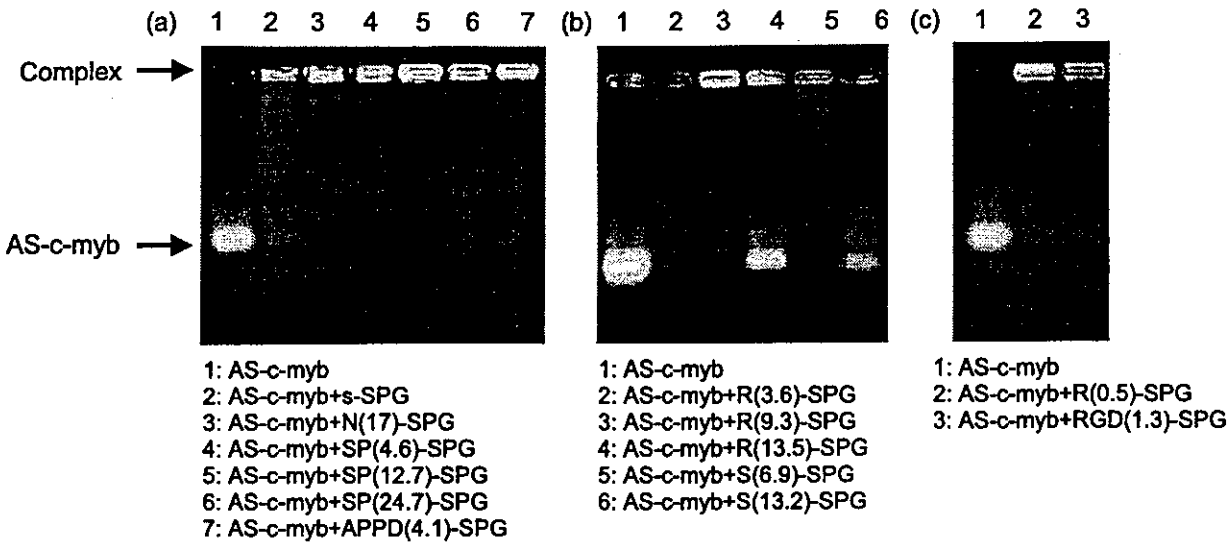


Fig. 4. Confirmation of the complex formation between AS-c-myb and the modified s-SPG samples by gel electrophoresis. 2 wt.% NuSieve agarose gel (BMA) was used and the gel was stained with GelStar® (BMA). As shown in Refs. [20,29,47], when the complex is formed, the migration does not occur and the complex stays at the starting hole.

of two s-SPG-repeating units and three base units, thus the stoichiometric ratio is 2:3. The molar ratio to give the minimum in the cell growth is different from the stoichiometry; however, the reason is not clear at this moment. On this composition, the cation/anion ratios in the complex are presented in the fourth column in Table 1. As shown by the table, except for the SP(24.7)-SPG system, the cation/anion ratio is less than 1.0, thus the overall charge of the complex is negative. Although we did not measure the net charge of the final complex, all ODN molecules are incorporated into the complex in this condition [20,47], thus the net charge should be estimated from the cation/anion ratio.

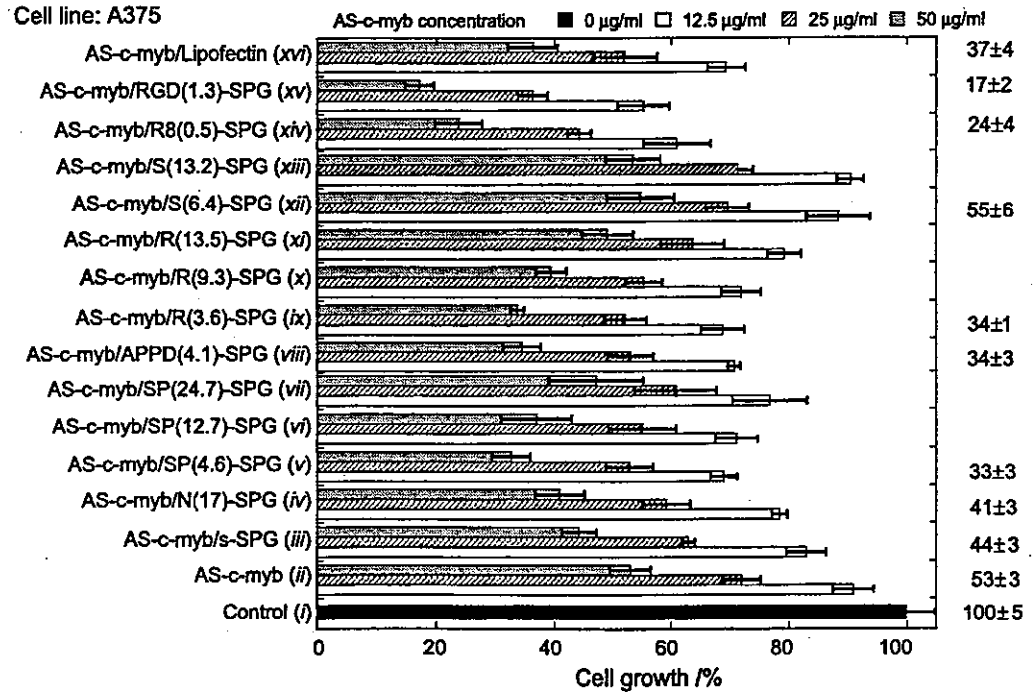


Fig. 5. Comparison of the cell growth for all samples, including the Lipofectin and unmodified s-SPG systems, assayed for the A375 cell lines. The numerical results of the proliferation for 50 μg/ml dose are presented in the left side of the histograms. The cell numbers were determined with Cell Counting Kit-8® (Dojindo, Japan), after we administrated AS-c-myb with various carriers and incubated the cells for 3 days. The AS-c-myb doses were 12.5, 25 and 50 μg/ml (unfilled bar, hatched bar and filled bar, respectively). The cell growth is defined by the number of cells normalized by that of the control (without any addition of AS-c-myb nor s-SPG, (i)). The s-SPG/AS-c-myb molar ratio was fixed at 1:1 [29].

When we exposed the cells to AS-c-myb itself, that is naked AS-c-myb, ((ii) in Fig. 5), the cell growth decreases with increasing the AS-c-myb dose. This decrease should be mainly ascribed to the antisense effect; however, some percentage of the cell death may be caused by cytotoxicity of the phosphorothioates. This issue will be discussed later in Fig. 6. When we added AS-c-myb as a complex with the unmodified s-SPG (iii) and compare the results with the same dose in the naked AS-c-myb, the cell growth is lower than that of the naked AS-c-myb. The difference is about 5–10% and can be ascribed to the advantage that the complex prevents AS-c-myb to form unfavorable interactions with the proteins in the culture medium. Thus, the complex can increase the cellular uptake efficiency [29]. When we exposed the cells to AS-c-myb as the modified s-SPG complexes having various cations such as aminoethanol (iv), spermine (v–vii) and *N,N′*-bis(3-aminopropyl)-1,3-propanediamine (viii), there is no significant improvement in the cellular uptake (i.e., no significant depression of the cell growth). When we compare the growth carefully, the introduction of these cations may decrease the efficiency by no more than 10%. Azzam et al. [18] reported that spermine can be introduced into dextran (about 10 mol%, 1 of 10 main chain glucoses) with the similar method with ours and the resultant polymer shows considerable enhancement in cellular uptake of plasmid DNAs. However, our cases (v–vii) do not show such enhancement. On the contrary, the antisense effect seems to become diminished with increasing the spermine modification rate (compare (v) and (vii)). By the way, when we mixed AS-c-myb and SP(24.7)-SPG, we observed turbidity. The main chain of s-SPG consists of β-(1 → 3)-D-glucan and it is usually less soluble than dextran which has a soluble α-(1 → 6)-glucan main chain. Therefore, we presume that the ion-pair formation between the AS-c-myb anion and the spermine cation may reduce the solubility of the complex. Furthermore, AS-c-myb may be bound too tightly to be released from the complex. This compaction problem is sometimes observed for the PEI/AS ODNs complex [3], while plasmid DNA hardly has such a problem in the PEI complexes.

When we examined the Arg attached systems (ix–xi), there was no significant difference from the other cation-attached s-SPG systems. This feature indicates that mono Arg simply acts as a cation and does not induce the specific effect on the poly Arg modified carries, which Futaki et al. [32] observed for octa- or branched-chain Arg systems. With increasing the Arg modification level, the antisense effect is decreased, due to poor solubility of the higher modified s-SPG as motioned in the gel-electrophoresis section. When AS-c-myb was added as the Ser attached s-SPG complexes (xii, xiii), the results are almost the same as the unmodified s-SPG system. This feature is reasonable because Ser is expected to act as an anion in our conditions [40], so that this complex should have no binding site for the cell.

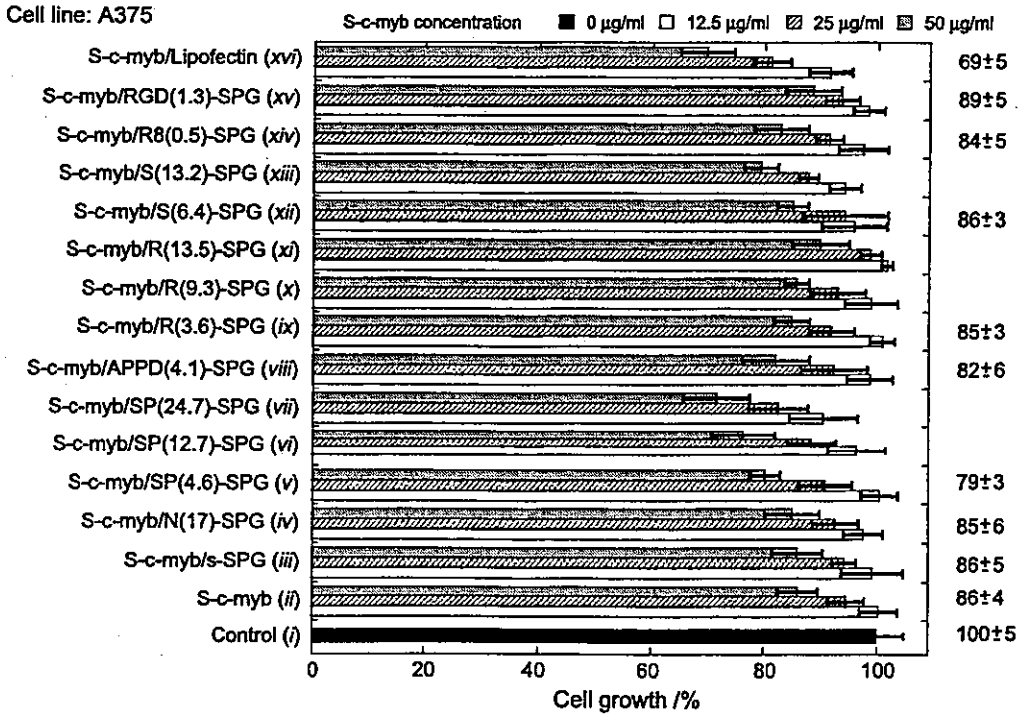


Fig. 6. Comparison of the cell growth for all samples, when we administrated the sense sequence (S-c-myb) instead of the antisense sequence (AS-c-myb). The other conditions were the exactly same as those in Fig. 5. The numerical results of the proliferation for the 50 μg/ml dose are presented in the left side of the histograms.

Dramatic depression in the cell growth is observed when AS-c-myb was administrated as the peptide attached s-SPG complex (xiv, xv). At 12.5 μg/ml dose, the cell growths are depressed to 60% for R8(0.5)-SPG and to 56% for RGD(1.3)-SPG. When the administration was increased to 50 μg/ml, the growths are drastically reduced to 24% for R8(0.5)-SPG and to 17% for RGD(1.3)-SPG. As discussed later in Figs. 6 and 8, the drastic depression can never be ascribed to the cytotoxicity of either phosphorothioates AS-c-myb or the peptide attached s-SPG. It should be emphasized that the very small amount of the modification level seems enough to enhance the cellular uptake efficiency. As mentioned in the strategy section, the RGD(1.3)-SPG complex is considered to be ingested through receptor-mediated endocytosis. On the other hand, the R8(0.5)-SPG complex is ingested through 'potocytosis' like pathway.

As presented in Table 1, all complexes studied are negatively charged in total because of the excess amount of AS ODNs. Therefore, the attractive Coulombic force should not work when the complexes approach to the negatively charged cell surface from far away. However, once the complexes happen to be in close vicinity to the cell surface by the thermal motion or the flow of the culture medium, the attached cations can interact with the cell surface. It is interesting that some negatively charged complexes can show comparable efficiency in the cellular uptake with the positively charged Lipofectin (see below). Negatively charged complexes should show a different performance from the conventional positively charged carries, such as longer circulation in the blood. Therefore, we can propose that this is one of the peculiar features of our system, which is different from the conventional systems.

We administrated AS-c-myb with Lipofectin according to the standard protocol in the Invitorogen manual [48] (xvi). The cell growth is less than those for amino- and α-amino acid appended s-SPG systems; however, it is relatively higher than the peptide appended s-SPG systems. According to the previous work, when RGD was incorporated into cationic liposomes for plasmid DNA delivery, the efficiency increased by 2–10 times [31]. Although exact comparison is impossible, our peptide appended s-SPG can be comparable with those functional liposomes.

Fig. 6 compares the cell growth when the sense sequence (not antisense sequence, denoted by S-c-myb) was administrated with the same conditions as those in Fig. 5. Theoretically speaking, there should be no depression observed in the cell growth. However, the cytotoxicity of the phosphorothioates decreases the growth due to non-antisense effect. From (ii) administration of the naked S-c-

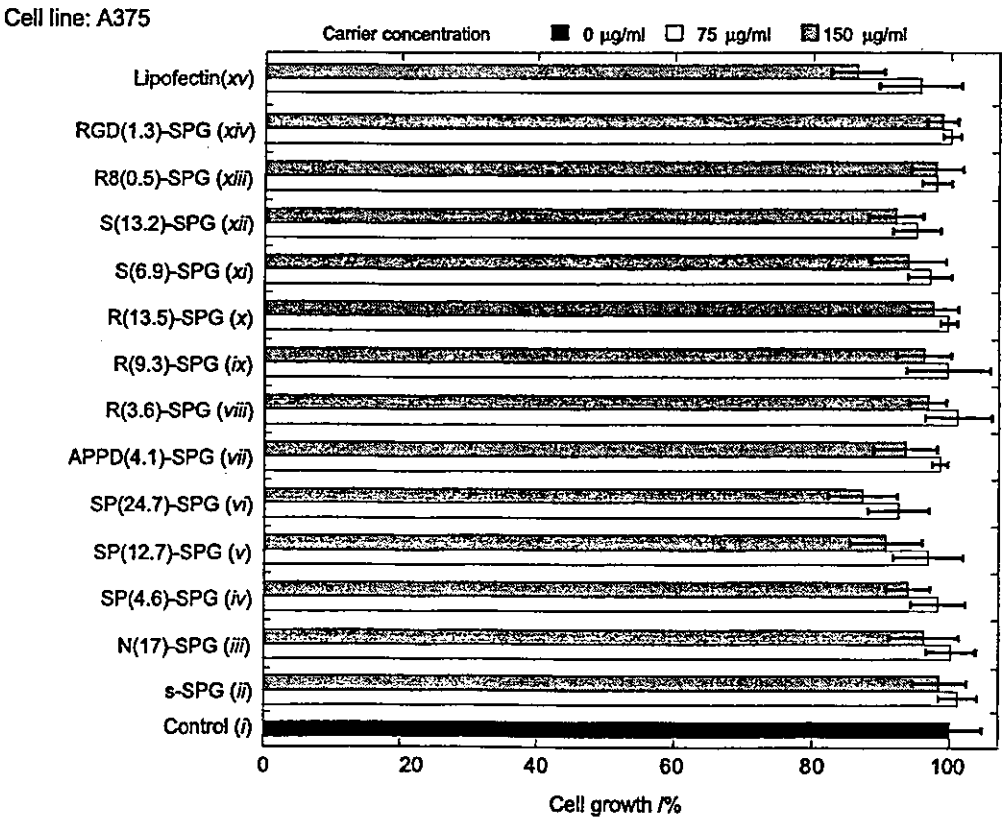


Fig. 7. The cytotoxicity test for the carriers used in this study. The doses are 75 and 150 μg/ml, and the 150 dose corresponds to the same amount of dose in weight as those in 50 μg/ml dose in Fig. 5.