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β-1,3-Glucan/antisense oligonucleotide complex stabilized with phosphorothioation and its gene suppression

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ABSTRACT

Most of antisense oligonucleotides (ASOs) subjected to current clinical evaluation belong to phosphorothioate (PS) analogues. Although PS has great advantage in DNase resistance, it can induce nonspecific side-effects. Thus it is important to investigate the influence of ASOs with different PS contents. In this paper, we prepared the complex consisting of schizophyllan (SPG) and ASOs attached a dA_{40} tail with different PS contents to the 3′ end of the ODN, which is introduced to stabilize the complex with SPG. With increase of PS content in the dA_{40}, its complexation ability with SPG was improved and the complex showed high thermal stability. The thermal stability of the fully phosphorothioated ASOs was obtained by only replacing 20% of the oxygen of the phosphodiester moiety. The ability of gene suppression between PS and phosphodiester for antisense sequences was almost the same, indicating that the antisense sequences need not to be PS backbone. These data may provide new insight for the interaction between β-1,3-glucan and DNA and help to deliver therapeutic ODNs.

1. Introduction

The concept of antisense therapy was proposed in the late 1970s by Zamecnik and Stephenson, who were the first to find that a specific short DNA sequence complementary to a particular part of the target mRNA can inhibit the corresponding protein expression [1]. Such biofunctional DNAs are denoted antisense oligonucleotides (ASOs) and several ASOs are currently examined in the advanced preclinical and clinical trials in the areas of HIV/AIDS and cancer [2,3]. Throughout the past studies, it is shown that poor cellular entry of ASOs and its vulnerability against DNase are crucial to execute its activity *in vivo*. To make the biological effects appreciable *in vivo* tests, overdose administration to animal models, such as more than 10 mg/kg, is sometimes conducted [4]. However, overdosed ASOs may induce toxicity and side-effects and, primarily, it is not practical in terms of the commercial and medical standpoints. In order to overcome such drawbacks, mainly, there are two approaches proposed: nucleotide analogues [5,6] and targeting delivery [7,8].

Among various nucleotide analogues, phosphorothioate (PS) ASO, in which one oxygen atom of the phosphodiester (PO) moiety is replaced by sulfur, have improved nuclease resistance and cellular uptake [9,10]. On the other hand, as a serious drawback, it has been shown that when PS ASOs are administrated at high concentrations, they induce nonspecific effects such as inhibition of proliferative activity [11,12]. This non-antisense effect is pointed out caused by the ability of PS molecules to bind particular cellular proteins [13,14]. Galderisi et al. showed that when ASOs was partially phosphorothioated, they showed dose-dependent and sequence specific inhibitions of target mRNA with less side-effects, while the fully phosphorothioate one showed a reduction of cell proliferation rate [12]. This finding suggests that the extent of PS modification may play an important role in inducing side-effects.

A natural polysaccharide schizophyllan (SPG) can be used as a delivering vehicle targeting to antigen presenting cells by use of the recognition ability of dectin-1 [15,16]. SPG is a member of the β-1,3-glucan family and have been known to exist as a right-handed triple helix in neutral water and as a single chain in alkaline solution (>0.25 N NaOHaq) [17]. When the alkaline solution of SPG is neutralized to pH 7–8, the single chain retrieves its original triple helix owing to the hydrophobic and hydrogen bonding interactions. In the presence of a particular polynucleotide such as poly(C) or poly(dA) in this neutralization process, two main-chain glucoses and one base of the polynucleotide form a stoichiometric complex [18–20]. Contrary to these long homo sequences, short hetero-sequences, such as antisense or CpG DNA do not form the complex with SPG. Since longer (dA) chains form more stable complex than shorter ones [21], we attached $(dA)_X$ tail to the 3′ end of ASO, where $X > 30–40$.

We reported that PS can form more stable complexes with SPG than PO and thus the PS complexes shows more prominent effect than PO ones [22]. When we use PS complexes, there is possibility

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to induce PS-originated side-effects. Since the previous studies indicate less toxicity for partial PS modification in ASOs, we decided to study the relationship between the complex stability and the PO/PS content in the dA tail and then evaluate the antisense efficiency *in vitro* by use of the primary macrophages with dectin-1 on their surface.

2. Material and methods

2.1. Materials

Schizophyllum commune-derived SPG ($M_w = 1.5 \times 10^5$, based on gel-permeation chromatography coupled to multiangle light scattering analysis) was kindly provided by Mitsui Sugar Co., Ltd. (Tokyo, Japan). All phosphorothioate ODN samples were synthesized at FASMAC Co., Ltd. (Kanagawa, Japan) and purified with high-performance liquid chromatography.

2.2. Preparation of phosphorothioate DNA complex with SPG

SPG was dissolved in 0.25 N NaOHaq for more 2 days to dissociate triple helix to single chain. Appropriate amount of SPG solution, ODN in water and phosphate buffer solution (330 mM NaH_2PO_4, pH 4.7) were mixed. After mixing, the mixture (ODN 60 μM, pH 7.4) was stored at 4 °C over night. The molar ratio ([SPG]/[ODN]) was controlled to 0.27.

2.3. Polyacrylamide gel electrophoresis (PAGE)

ODN (20 ng) or its complex with SPG were separated by a 15% acrylamide gel for 1 h at 100 V. After the gel was stained with SYBR Gold (Invitrogen, Carlsbad, CA), the fluorescent image was obtained using a PharosFX (Bio-Rad, Richmond, CA).

2.4. Gel-permeation chromatography (GPC)

GPC was carried out using a Showa Denko DU-H2130 pumping system (Tokyo, Japan) at the flow rate of 0.8 ml/min with OHpak SB802.5 and OHpak 806 columns (Showa Denko Co., Ltd.). 0.1 M phosphate buffer (pH 7.4) containing 0.5 M KCl was used as a mobile phase. The elute was detected by a multiangle light scattering (LS) detector (DAWN-HELEOS, Wyatt Technology, Santa Barbara, CA), an UV detector (SPD-10A; SHIMADZU Co., Ltd, Kyoto, Japan) and a refractive index (RI) detector (RI-71S; Showa Denko). RI and LS signals were used to calculate the weight-average molecular mass (M_w) according to the instruction manual for Dawn-HELEOS. The refractive index increment (d*n*/d*c* value) was 0.157 ml/g.

2.5. Circular dichroism spectroscopy

The circular dichroism in 240–320 nm regions was measured on a Jasco J-802 spectropolarimeter (JASCO, Tokyo, Japan) at 20–80 °C using a 1 cm quartz cell with a water jacket.

2.6. Animal works

Animal experiments were performed according to Guidelines for Animal care and Use committee, Kyushu Institute of Technology. Thioglycolate-elicited macrophages were isolated from C57BL/6 mice (Kyudo Co., Ltd., Kumamoto, Japan) by standard procedure [23]. In brief, mice were injected intraperitoneally with 3 ml of 3% thioglycolate medium. Three days later, peritoneal macrophages were harvested by peritoneal lavage using 6 ml of RPMI 1640. Lavage fluids were pooled and centrifuged at 100*g* for 5 min. Primary macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin at 37 °C in 5% CO_2.

2.7. Cytokine assay

Thio-Mφs were plated at 5×10^4 cells/well in 96-well plates, and treated for 24 h. The cells were added ODN/SPG complex or ODN (0.5 μM) and incubated for 30 min at 37 °C, and then incubated for 5 h at 37 °C in fresh medium containing LPS (10 ng/ml). Levels of TNF-α released into the supernatants were measured by using Murine TNF-alpha ELISA Development Kit (PeproTech, Rocky Hill, NJ).

3. Results and discussion

3.1. Complex formation between SPG and $(dA)_X$

Fig. 1A shows the sequence length dependence of the complexation for PS-dA_X with polyacrylamide gel electrophoresis (PAGE). There is no free $(dA)_X$ observed at $X > 30$ after complexed with SPG, while there is still small amount of free $(dA)_{20}$ for $X = 20$. When a biologically substantial hereto-sequence such as an antisense TNF-α with 20mer was attached to $(dA)_{20}$, the yield becomes less than $(dA)_{20}$ itself (compare the second lanes between Fig. 1A and B). When the tail became longer, the AS-dA_{30} and -dA_{40} shows almost no free $(dA)_X$. These results showed that the longer dA tail gives the larger yield and the attached hetero-sequence decrease the yield. This tail length effect in yield can be rationalized by the complex stabilization due to the cooperative polymeric effect.

We prepared six dA_{40} samples with different PS contents: 0/39 PS, 8/39 PS, 13/39 PS, 20/39 PS, 26/39 PS and 39/39 PS (see Table 1 for the substitution sites), where the denominators indicate the number of the PS linkages. The complexes from them were prepared with the established method (see Section 2). As shown by PAGE image in Fig. 2A, there was no free DNA observed for the 13/39, 20/39, 26/39 and 39/39 PS complexes, while and 8/39 PS showed some amount of free $(dA)_X$. The yields were determined to be 90% and 70% for 8/39 and 0/39 PS, respectively. Fig. 2B compares the gel permeation UV (260 nm) chromatograms between 39/39 and 0/39 PS after complexed. From the peak area of the complex (around 16–20 min) and free ODN (around 20–25 min), the yields were determined to be 90% for 39/39 and 60% for 0/39 PS, respectively, being consist with the PAGE results. The above results conclude that at most 30% of phosphorothioation gives the same yield as 100 mol.% as well as confirming that PS forms more stable complex than PO.

The relation of phosphorothioation and the complex stability is interesting and may be related to the molecular structure of the complex. Computational chemistry revealed that the hydrogen bonding between the SPG main-chain glucose and the base moiety is the major driving force for the complexation, especially the cooperative arrayed hydrogen bonding along the chains stabilizes the complex [24]. Since the phosphodiester moiety links the adjacent two riboses and determines the tilting angle and stacking nature of the adjacent two dA rings, PS may provide a more favorable configuration to form such hydrogen bonding than PO. The spatial relation between the adjacent two dA rings can be sensitively reflected to circular dichroism (CD).

3.2. Thermal stability of complexes

Fig. 3A showed the CD spectra in the range of 240–320 nm for all PS and PO $(dA)_{40}$ samples at 20 °C. Overall spectral shapes were almost same for all samples, although with increasing the PS content, the negative peak at 248 nm slightly increased in intensity.

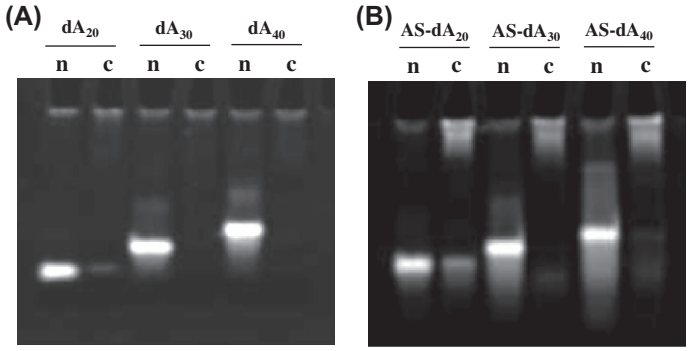

Fig. 1. Polyacrylamide gel electrophoresis migration patterns for complexed (c) and naked (n) dA_{20}, dA_{30}, dA_{40} samples (A) and AS-dA_{20}, AS-dA_{30}, AS-dA_{40} samples (B).

Table 1
Sample codes and the sequences.

Sample code	DNA sequence[a]
0/39	A_{40}
8/39	$(AsAAAA)_8$
13/39	$(AsAA)_{13}A$
20/39	$(AsA)_{20}$
26/39	$(AsAsA)_{13}A$
39/39	$(As)_{39}A$

[a] The small letter "s" indicates the phosphorothioate (PS) linkage.

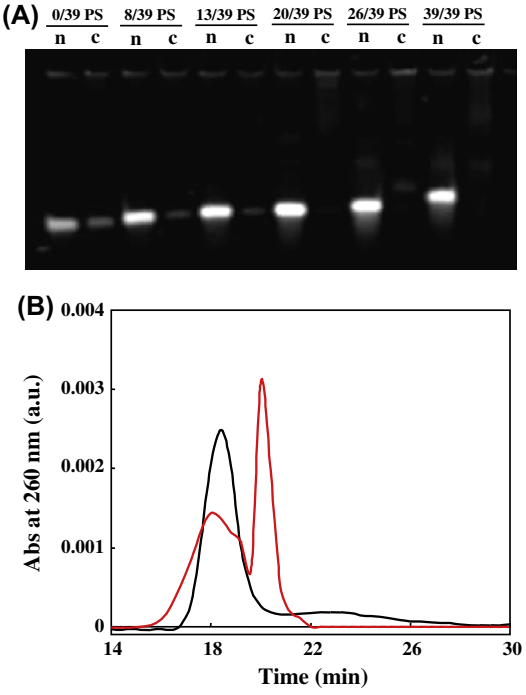

Fig. 2. Polyacrylamide gel electrophoresis migration patterns for complexed (c) and naked (n) dA_{40} samples with different PS contents (A). Gel permeation chromatograms of the complex consisting of SPG and AS-ODN with PS (black line) and PO (red line) (B). Chromatograms detected by UV absorbance at 260 nm are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Since this wavelength range is related to the induced CD between the base moieties, the similarity in CD indicates that all $(dA)_{40}$ take the same conformation. Upon the complexation, the CD spectra is drastically changed as presented in Fig. 3B, where for convenience, the naked and complexed PO-dA_{40} are compared. This means that dA adopts a new conformation to form the SPG complex, as reported previously (Ref. [18,19]). When compared among the complexed spectra, the four complexes from 0/39 PS to 20/39 PS showed almost identical ones. With increasing the S content to 26/39 and 39/39, they showed different one from others. For 39/39 PS, the CD value at 248 nm decreased and that of 264 nm increased. These results agree qualitatively with the above mentioned speculation that the PS can provide a more preferable conformation for the complexation than PO. However, quantitatively, there is discrepancy in CD and PAGE results, where 33% is enough to reach 100% yield while more than 66% is necessary to change CD.

Fig. 3C plots the CD intensity at 282 nm against the temperature. As mentioned above, at low temperatures, the CD intensities for the complexes are larger than naked ones. Within the certain temperature range, the intensities for the complexes decreased and merged into those for corresponding dA_{40} samples. From this transition, the dissociation (or melting) temperature T_m is plotted in Fig. 3D. With increasing the PS content from 0/39 to 8/39 PS, T_m increased from 49 to 59 °C. Further increase of PS content did not increase T_m. These results confirm that the PS backbone modification induces more stable complex than PO backbone, while only 20 mol.% modification should be enough to give the same increment in T_m with 100% one.

3.3. Gene silencing by complex

We prepared five ASOs samples in which a PO TNF-α antisense sequence, which has been well demonstrated in previous work (AACCCATCGGCTGGCACCAC [25]), is attached a dA_{40} tail at the 3′ end with different S contents as well as fully phosphorothioated one (see Table 2). PAGE images for all complexes are presented in Fig. 4. For the AS-0/39 PS complex, there was a large amount of the free ASO and the complexation ability with SPG was determined to be 50%, again this value is smaller than that of the 0/39 PS $(dA)_{40}$ tail itself. With increase of the S content, the free ASO band disappeared, indicating increase of the complexation ability with SPG. The AS-13/39 complex showed no free band, similarly with the result of dA_{40} itself.

We carried out the TNF-α gene silencing effect by use of thus prepared ASOs samples as follows. The SPG complex can be incorporated into the cells via dectin-1, which is a major receptor to recognize β-1,3-glucans on many antigen presenting cells (APCs), including macrophages and dendritic cells [15,16]. After prepared thioglycolate-elicited peritoneal macrophages (thio-Mφ), we applied the ASO/SPG complexes (1 μM) to them and subsequently stimulated LPS with 10 ng/ml to secret TNF-α [26]. The amount of secreted TNF-α is plotted against the PS content (Fig. 5). All of the naked doses did not reduce the secretion. On the other hand, as expected, the SPG complex showed the TNF-α silencing. With

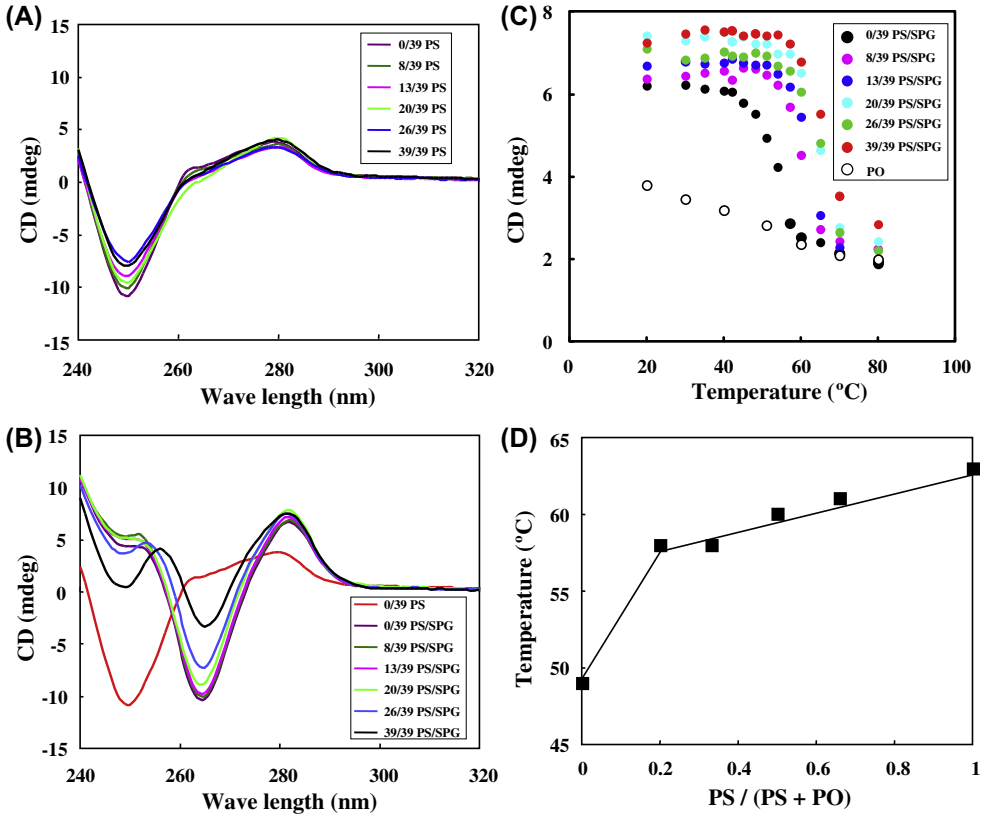

Fig. 3. PS content dependence of the CD spectrum of the naked (A) and complexed (B) dA_{40} measured at 20 °C. Temperature dependence of the CD intensity at 282 nm for the complex (C). T_m for dA40 with different PS contents (D).

Table 2
Sample codes and sequences.

Sample code	DNA sequence[a]
AS-0/39	AS-A_{40}
AS-8/39	AS-$(AsAAAA)_8$
AS-13/39	AS-$(AsAA)_{13}A$
AS-20/39	AS-$(AsA)_{20}$
AS-39/39	AS-$(As)_{39}A$
All PS	(AS)s-$(As)_{39}A$

All PS has PS linkages in the antisense part and other samples do not.
The sequence of antisense for TNF-α is AACCCAT CGGCTGGCACCAC.
[a] The small letter "s" indicates the phosphorothioate (PS) linkage.

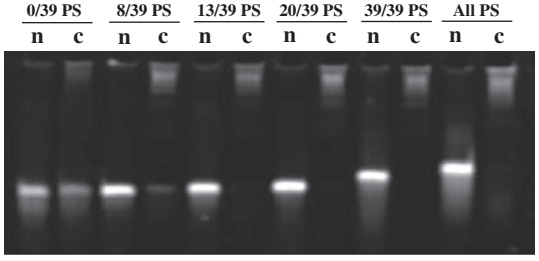

Fig. 4. Comparison of the gel electrophoresis migration patterns of the complexes consisting of SPG and ASOs-dA_{40} with different PS contents. n and c represent naked ASO and its complex with SPG, respectively.

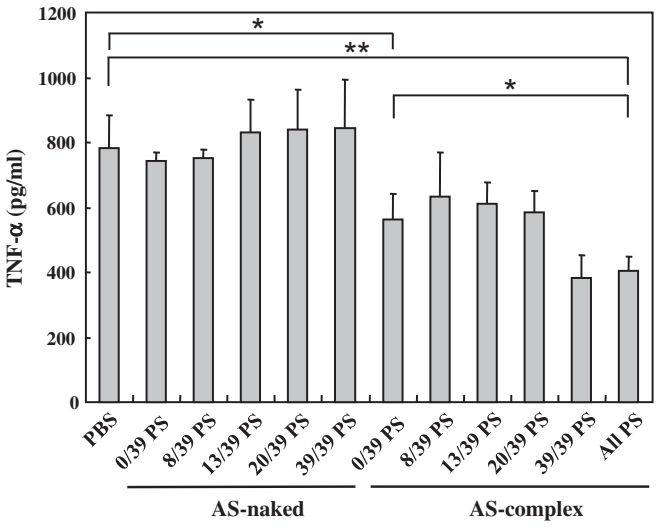

Fig. 5. Comparison of the LPS-induced TNF-α secretion after treated with ASO/SPG complexes. Results are mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

increasing the PS content, the silencing effect was slightly enhanced. This is probably the higher PS complex is more stable in the medium and taken up more easily by the cells. The ability of suppression between PS and PO for antisense sequences (All PS and AS-39/39, Table 2) was almost the same, indicating that the antisense sequences need not to be PS backbone.

4. Conclusions

Our results demonstrate that the complex with the phosphorothioate dA_{40} showed thermally more stable than phosphodiester

dA_{40}, and the stability increased to the same level as the fully phosphorothioated one by only replacing 20–30 mol.% of the oxygen of the phosphodiester moiety. The ability of antisense gene suppression between PS and PO for was comparable. These findings may provide new insight for the interaction between β-1,3-glucan and DNA and help to deliver therapeutic PS oligonucleotides with less side-effect.

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