Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 327–330
Bioorganic & Medicinal Chemistry Letters

Schizophyllan–folate conjugate as a new non-cytotoxic and cancer-targeted antisense carrier

Teruaki Hasegawa,[a] Tomohisa Fujisawa,[a] Shuichi Haraguchi,[a] Munenori Numata,[a] Ryouji Karinaga,[b] Taro Kimura,[c] Shiro Okumura,[c] Kazuo Sakurai[b] and Seiji Shinkai[a,*]

[a]*Department of Chemistry, Graduate School of Engineering, Kyushu University, Fukuoka 812-8581, Japan*
[b]*Department of Chemical Processes and Environments, Faculty of Environmental Engineering, The University of Kitakyushu, Kitakyushu 808-0135, Japan*
[c]*Fukuoka Industrial Technology Center, Biotechnology and Food Research Institute, Kurume 839-0861, Japan*

Received 28 September 2004; revised 23 October 2004; accepted 25 October 2004
Available online 13 November 2004

Abstract—Schizophyllan having folate-appendages was synthesized from native schizophyllan through $NaIO_4$-oxidation and the subsequent reductive amination in aqueous ammonia followed by amido-coupling with folic acid. The resulting folate-appended schizophyllan can form stable complex with poly(dA), show specific affinity toward folate binding protein, and mediate effective antisense activity in cancer cells.

Effective, safe, and low cost delivery systems are essential to establish practical antisense therapy.[1] At the very early stage of investigation, viral vectors were thought to be promising,[2] however, little viral vector has been used in practical therapy so far because of their inherent danger including its strong immunogeneity and possible mutation. Increasing research efforts have been, therefore, placed on developing artificial carriers (liposomes,[3] linear polymers,[4] dendrimers,[5] etc.) which mostly utilize their inherent polycationic nature for complexation with polyanionic antisense. However, since polycationic nature itself induces strong cytotoxicity, no artificial carrier so far reported satisfies all the requisites for practical delivery systems.

Schizophyllan (SPG), β-1,3-glucan having β-1,6-glucoside-appendages at every three repeating units, has been received great interest because of its reversible coiled-helix transition.[6] As illustrated in Figure 1, SPG has homo triple-helix structure (*t*-SPG) in water whereas it is dissociated into random coiled single strands (*s*-SPG) in dimethylsulfoxide (DMSO). Recently, we found that when *s*-SPG in DMSO is mixed with certain homo polynucleotides in water, unique hetero triple-stranded macromolecular complexes consisting of two SPG strands and one polynucleotide strand are formed.[7] This phenomenon is of quite interest, since such SPG/polynucleotide complexes are formed not through the conventional polyelectrostatic interactions but through novel 'shape-fitting' interactions between helical SPG and helical polynucleotides. Since no cationic nature is required for the complex formation, SPG should be a potent candidate to overcome the aforementioned conventional dilemma.

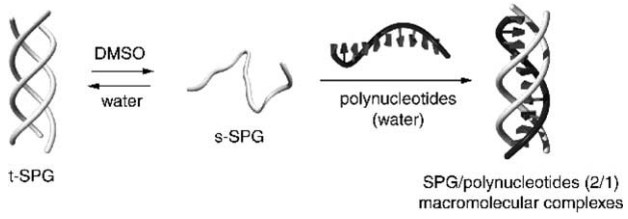

Figure 1. Helix-coil transition of schizophyllan (SPG) and formation of SPG/polynucleotides macromolecular complexes.

In the series of our intense research, we found various advantages of SPG as antisense carriers, that is, (1) excellent thermal stability of the complex under the physiological conditions,[8] (2) long blood circulation time of the complex owing to the lack of β-1,3-glucanase

Keywords: Schizophyllan; Antisense delivery system; Cancer; Folic acid.
* Corresponding author. Tel.: +81 92 642 3585; fax: +81 92 642 3611; e-mail: seijitcm@mbox.nc.kyushu-u.ac.jp

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.10.071

in the mammals,[9] (3) protection of the complexed polynucleotides against degradation by DNases,[10] and (4) quick release of the complexed polynucleotides to hybridize with target RNA.[11]

Our next step is to develop cancer-targeted antisense carriers, by which the complexed antisense oligonucleotides (AS-ODNs) are delivered specifically into cancer cells. Such specificity is quite important to establish practical cancer therapy, since it can increase effective antisense concentrations in the cancer cells and decrease required doses of antisenses to reduce total costs. Furthermore, such cancer-specificity can also minimize unfavorable uptake of antisenses by nontarget cells to reduce burdens on the patient's body. The most potential strategy to develop such delivery systems includes conjugations of SPG with cancer-specific ligands, such as folate.[12,13] Since cancer cells over express folate binding proteins (FBP) on its cell surfaces to uptake folic acid and the other folate-related molecules, SPG carrying folate-appendages (SPG-FA, Chart 1) should be a potent candidate for cancer-targeted antisense carriers.

SPG-FA was synthesized from native SPG (M_w = 150 kDa) that was first converted to aldehyde functionalized SPG through 1,2-diol-selective oxidation by treatment with aqueous $NaIO_4$.[14] β-1,6-Glucoside-appendages were selectively modified in this oxidation step whereas SPG mainchain having no 1,2-diol remains intact. This appendage-selective oxidation is quite essential to develop various SPG-based antisense carriers having cell-targeting potency, since structural and conformational perturbation on the mainchain should destabilize the resulting carrier/AS-ODNs complexes. The subsequent reductive amination using aqueous ammonia and $NaBH_3CN$ afforded amino functionalized SPG, which was finally coupled with folic acid in the presence of DCC and Et_3N to afford SPG-FA. The weight-averaged molecular weight (M_w) was estimated to be 90 kDa, that is slightly decreased in comparison to that of native SPG. The conversion (n in Chart 1) was estimated to be 0.09 based on absorbance at 283 nm which is characteristic for the folate-appendages.[15] We assumed that less hindered terminal-carboxylates should be preferentially used for the coupling, although we have no structural evidence to support our assumption.

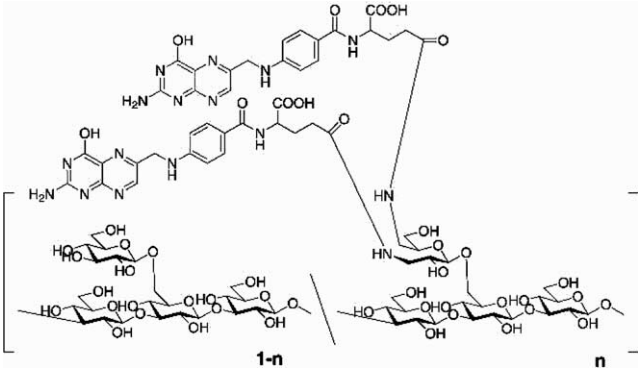

Chart 1. Structure of schizophyllan-based antisense carrier (SPG-FA) presenting folate-appendages as targeting signals for cancer cells.

The macromolecular complexes composed of SPG-FA and polynucleotides can be easy prepared by mixing SPG-FA in DMSO with polynucleotides aqueous solution.[16] Formation of the macromolecular complexes was confirmed by circular dichroic (CD) spectra. As shown in Figure 2a, SPG-FA/poly(dA) complex shows a CD spectrum, in which a predominant negative peak (250 nm) observed for free poly(dA) is suppressed and new negative (265 nm) and positive (282 nm) peaks appear. This CD spectral change is similar to that of SPG/poly(dA) complex, indicating the formation of a hetero triple-stranded macromolecular complex consisting of two SPG-FA strands and one poly(dA) strand.

One apparent difference between the CD spectra of SPG-FA/poly(dA) complex and SPG/poly(dA) complex is a strongly enhanced positive CD signal of the former

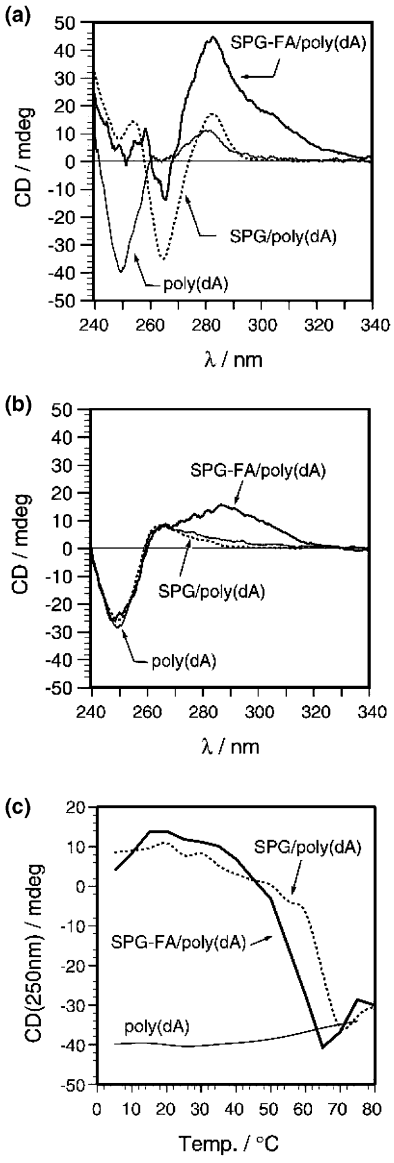

Figure 2. CD spectra of SPG-FA/poly(dA) complex, SPG/poly(dA) complex, and free poly(dA) at (a) 5 and (b) 75 °C and (c) temperature-dependence of their CD intensities (250 nm): d = 0.5 cm, [SPGs] = 0.43 mg/mL, [poly(dA)] = 0.083 mg/mL, 0.83 mM Tris–HCl buffer (pH 8.0) containing 8.3% v/v DMSO.

at around 280 nm which should arise from the folate-appendages. This assumption was supported by a positive CD signal of SPG-FA/poly(dA) complex at 75 °C (Fig. 2b), at which SPG-FA/poly(dA) complex is dissociated into the individual strands. Since (1) no or negligibly weak CD signal was observed at this wavelength for the liberated (or free) poly(dA) and (2) folate-appendages have a significant adsorption band at this region (λ_{max} = 283 nm), this spectral data clearly indicate that the folate-appendages attached onto chiral polysaccharide are origins of this enhanced positive CD signal.

We measured the CD spectra at various temperatures (5–80 °C) to assess thermal stabilities of the complexes. The CD spectra of SPG-FA/poly(dA) complex are independent of the temperature up to 40 °C, which are suddenly changed with increasing temperature at around 50 °C into the CD spectra attributable to free poly(dA). This CD spectral change proves that the complex is dissociated cooperatively at this temperature. SPG-FA/poly(dA) complex showed the melting temperature (T_m) at around 50 °C (Fig. 2c), which is lower than that (63 °C) of SPG/poly(dA) complex but still sufficiently higher than the physiological temperature.

Specific FBP-affinity of SPG-FA/poly(dA) complex was evaluated by using surface plasmon resonance (SPR) assay using Au-surfaces immobilized with FBP from bovine milk. Before the binding assays, commercially available sensor chips whose Au-surfaces are immobilized with carboxymethyldextran (Biacore, sensor chip CM5) were first treated with EDC/NHS in water to activate the carboxyl groups. FBP was then immobilized by coupling their amino groups with activated carboxyl groups on the resultant sensor chips. The residual activated carboxyl groups were finally deactivated by treatment with aqueous 2-aminoethanol to yield sensor chips having a FBP-immobilized Au-surface.

An injection of SPG-FA/poly(dA) complex induces a rapid increase in the resonance unit, indicating a binding of this complex onto the FBP-immobilized Au-surface (Fig. 3). On the other hand, such an increment is observed neither for SPG/poly(dA) complex nor for free poly(dA). We also confirmed that SPG-FA/poly(dA) complex has no affinity toward other proteins, such as bovine serum albumin (BSA). These data clearly demonstrate that the folate-appendages of SPG-FA/poly(dA) complex act as effective ligands to mediate the specific binding between SPG-FA/poly(dA) complex and FBP. One can expect therefore that SPG-FA/polynucleotide complexes can specifically bind onto over expressed FBP on the surfaces of cancer cells.

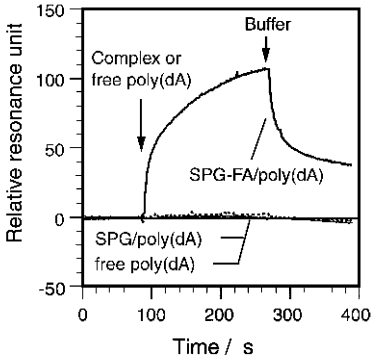

Figure 3. SPR sensorgrams obtained by (plane line) SPG-FA/poly(dA) complex, (dotted line) SPG/poly(dA) complex, and (thin line) free poly(dA): FBP-immobilized Au-surface, 25 °C, 80 mM Tris–HCl buffer (pH 8.0), 25 °C, Flow rate = 20 μL min^{-1}, [poly(dA)] = 0.54 mg mL^{-1}, [SPG-FA] or [SPG] = 2.7 mg mL^{-1}.

The association (k_a) and dissociation rate constants (k_d) of the specific binding were estimated to be $82.3 \pm 13.2\,M^{-1}s^{-1}$ and $9.92 \pm 1.95 \times 10^{-3}\,s^{-1}$, respectively, through computational curve fitting on the association and dissociation phases. The affinity constant ($K_a = k_a/k_d$) could be, therefore, estimated to be $4.63 \pm 0.52 \times 10^4\,M^{-1}$. It should be noted that we defined the concentration of SPG-FA/poly(dA) complex based on its nucleotide units. Since a folate/nucleotide ratio in the complex can be estimated to be 0.12 based on the conversion (n) and the stoichiometry of SPG-FA versus poly(dA) in the complex, the K_a value can be converted to $3.9 \pm 0.4 \times 10^5\,M^{-1}$ based on the folate-appendages. This K_a value is comparable to that ($K_a = 3.3 \times 10^5\,M^{-1}$) reported in the literature for a binding between monomeric folic acid and FBP under the neutral condition,[17] although the FBP-affinity of folate is intricate and various K_a values ($\sim 10^{10}\,M^{-1}$) have been also reported depending on experimental conditions (pH, [FBP], etc.).[18]

Antisense delivery mediated by SPG-FA was evaluated using KB human epithelial cells (KB cells) and SPG-FA/AS-ODN complex. In this assay, we used a complementary ODN (5′-GTGCCGGGGTCTTCGGGC-3′) that is well-known to bind to *c-myb* mRNA and to lead depression of *c-myb* and suppression of cell growth. Since our previous work revealed that SPG does not form the macromolecular complexes with short and hetero-ODNs, we used phosphorothioate-type ODN having $(dA)_{40}$-tag (5′-GTGCCGGGGTCTTCGGGC-$(A)_{40}$-3′, AS-*c-myb*).[19]

KB cells were cultured for three days in folate-free MEM containing SPG-FA/AS-*c-myb* complex, SPG/AS-*c-myb* complex, or free AS-*c-myb*, and then the cell-numbers were counted by using Cell Counting Kit-8 (Dojin). As shown in Figure 4a, SPG-FA/AS-*c-myb* complex suppresses the cell growth much more effectively than SPG/AS-*c-myb* complex as well as free AS-*c-myb*. SPG/AS-*c-myb* complex also shows the enhanced antisense efficiency in comparison to free AS-*c-myb*, suggesting that the complexed AS-*c-myb* is protected against enzymatic degradations by DNases and/or nonspecific associations with serum proteins. Together with this data, large increment of the antisense activity observed for SPG-FA/AS-*c-myb* complex is clearly ascribed to a synergistic effect of (1) protection of the complexed AS-*c-myb* and (2) enhanced cell affinity arising from the folate-appendages. The latter effect can be easily confirmed from Figure 4b and c, in which the antisense activity of SPG-FA/AS-*c-myb* complex is suppressed with increasing concentration of folic acid in the media. On the contrary, no such effect

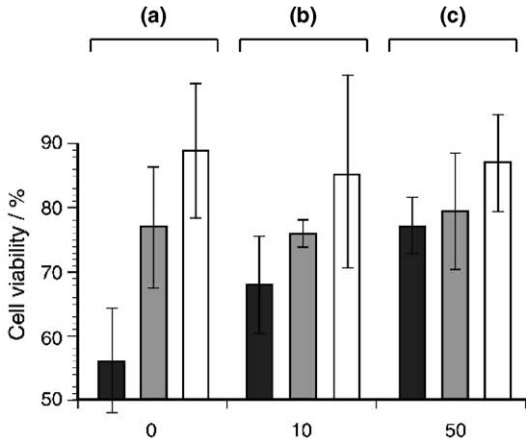

Figure 4. Relative cell growth (%) of KB cells after three days incubation with (black bars) SPG-FA/AS-*c-myb* complex, (gray bars) SPG/AS-*c-myb* complex, and (open bars) free AS-*c-myb*: MEM containing (a) 0, (b) 10, or (c) 50 μg mL^{-1} of folic acid, [SPG-FA/AS-*c-myb*], [SPG/AS-*c-myb*], and [AS-*c-myb*] = 50 μg mL^{-1} (nucleotide-fraction).

of co-existing folic acid was observed for SPG/AS-*c-myb* complex or free AS-*c-myb*. It should be noted that SPG-FA/AS-*c-myb* complex shows a substantial antisense effect even in MEM containing 10 μg mL^{-1} (ca. 20 μM, Fig. 4b) of folic acid. The FBP-affinity of SPG-FA/AS-*c-myb* is, therefore, expected in human body, in which the blood concentration of folate is only ca. 0.01 μM.[20]

We also carried out referential experiments using the corresponding scrambled sequence (SC-*c-myb*). As expected, SPG-FA/SC-*c-myb* complex as well as free SC-*c-myb* showed no or negligibly weak antisense activity (96.1 ± 9.8 and 98.3 ± 6.2 %, respectively), clearly proving that suppression of the cell growth observed for SPG-FA/AS-*c-myb* complex can be ascribed not to the cytotoxicity of SPG-FA but to the antisense activity of AS-*c-myb*. It should be emphasized here that the non-cytotoxicity of SPG-FA arises from its neutral nature that is one of the most attractive advantages of SPG-based antisense carriers over the conventional polycationic ones.

In conclusion, SPG-FA forms stable complex with poly(dA) and the resultant macromolecular complex shows the specific FBP-affinity. SPG-FA is also applicable to the complexation with an antisense carrying a poly(dA)-tail to enhance its antisense activity in KB cells. Along with many advantages of SPG as an antisense carrier, SPG-FA should be useful as a non-cytotoxic and cancer-targeting antisense carriers.

Acknowledgements

This work was supported by the Japan Science and Technology Corporation (SORST Program). We also thank Taito Co., Japan, for providing the native schizophyllan.

References and notes

1. Chirila, T. V.; Rakoczy, P. E.; Garrett, K. L.; Lou, X.; Constable, I. J. *Biomaterials* **2002**, *23*, 321.
2. Chatterjee, S.; Wong, K. K., Jr. *Methods* **1993**, *5*, 51.
3. Leamon, C. P.; Cooper, S. R.; Hardee, G. E. *Bioconjug. Chem.* **2003**, *14*, 738.
4. Kakizawa, Y.; Harada, A.; Kataoka, K. *Biomacromolecules* **2001**, *2*, 491.
5. Maszewska, M.; Leclaire, J.; Cieslak, M.; Nawrot, B.; Okruszek, A.; Caminade, A.-M.; Majoral, J.-P. *Oligonucleotides* **2003**, *13*, 193.
6. Chuah, C. T.; Sarko, A.; deslandes, Y.; Marchessault, R. H. *Macromolecules* **1983**, *16*, 1375.
7. Sakurai, K.; Shinkai, S. *J. Am. Chem. Soc.* **2000**, *122*, 4520.
8. Mizu, M.; Kimura, T.; Koumoto, K.; Sakurai, K.; Shinkai, S. *Chem. Commun.* **2001**, 429.
9. Nagasaki, T.; Hojo, M.; Uno, A.; Satoh, T.; Koumoto, K.; Mizu, M.; Sakurai, K.; Shinkai, S. *Bioconjug. Chem.* **2004**, *15*, 249.
10. Mizu, M.; Koumoto, K.; Kimura, T.; Sakurai, K.; Shinkai, S. *Biomaterials* **2004**, *25*, 3109.
11. Koumoto, K.; Mizu, M.; Sakurai, K.; Kunitake, T.; Shinkai, S. *Chem. Biodiversity* **2004**, *1*, 520.
12. Pan, D.; Turner, J. L.; Wooley, K. L. *Chem. Commun.* **2003**, 2400.
13. Zuber, G.; Zammut-Italiano, L.; Dauty, E.; Behr, J.-P. *Angew. Chem., Int. Ed.* **2003**, *42*, 2666.
14. $NaIO_4$ (0.3 equiv) was used for one 1,6-glucoside-appendages.
15. The conversion ratio (*n*) was estimated based on absorbance (ε = ca. 2965) of free folic acid in aqueous solution containing DMSO (14% v/v).
16. SPG-FA in DMSO (5 mg mL^{-1}, 100 μL) was mixed with polynucleotides in water (1 mg ml^{-1}, 100 μL) and then, diluted with Tris–HCl buffer (1 mM, pH 8.0, 1000 μL). The resultant solution was incubated for two days at 4 °C to yield the corresponding macromolecular complexes.
17. Salter, D. N.; Scott, K. J.; Slade, H.; Andrews, P. *Biochem. J.* **1981**, *193*, 469.
18. Nixon, P. F.; Jones, M.; Winzor, D. J. *Biochem. J.* **2004**, *382*, 215.
19. Matsumoto, T.; Numata, M.; Anada, T.; Mizu, M.; Koumoto, K.; Sakurai, K.; Nagasaki, T.; Shinkai, S. *Biochim. Biophys. Acta* **2004**, *1670*, 91.
20. Ford, E. S.; Bowman, B. A. *Am. J. Clin. Nutr.* **1999**, *69*, 476.