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Proposal of new modification technique for linear double-stranded DNAs using the polysaccharide schizopyllan☆

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Abstract—A natural polysaccharide schizophyllan (SPG) has been known to form a stable complex with poly(dA). We attached a poly(dA)$_{80}$ tail to the both ends of a linear double-stranded DNA, which had been prepared from a plasmid DNA vector. The poly(dA) tailed DNA verified to form complex with SPG by gel electrophoresis and atomic force microscopy (AFM). AFM images indicated that the complexes exhibit a dumbbell-like architecture, that is, quite similar to that of adenovirus genome. The complex demonstrated excellent exonuclease resistance, probably because of the protection effect by SPG complexation.

Nonviral methods of DNA transfer (i.e., nonviral vectors) have great advantage because of the absence of infectious or mutagenic capability. Therefore, future of gene therapy depends on emerging of good nonviral vectors, although most of them are still under development. In fact, nonviral vectors have many problems to overcome; among others, the poor transfection is a serious drawback. Transfection of nonviral vectors has been improved by imitating how viruses infect the host cells. Adenovirus is one of the best and realest models. The adenovirus core mainly consists of linear double-stranded genomic DNA, highly basic viral proteins VII and V, and the terminal protein.[1,2] Protein VII is the major protein component of the core and condenses the genomic DNA similar to histone. Compaction of DNA by cations is a good example to imitate Protein VII. The adenovirus terminal protein is covalently attached to the 5′ ends of the genome DNA and plays an important role in genome replication. Although not adenovirus proteins, the integrase protein of retrovirus (IN) is bound to the end of the liner retrovirus DNA and INs are essentially important for integration of the retrovirus DNA into the host chromosome.[3] All of those examples suggest that the modification of the end of liner double-stranded DNA is important to increase the efficiency of the DNAs in nonviral vectors. As far as we know, there are a few studies to specifically modify the end of double-stranded DNAs.[4–6]

Schizophyllan (SPG) is a natural β-(1→3)-D-glucan produced by the fungus *Schizophyllan commune* (Fig. 1 for the chemical structure).[7] SPG can form a macromolecular complex with some homo-polynucleotides, such as poly(C), poly(A), poly(U), poly(dA), and poly(dT).[8,9] Since this finding, we have applied this novel complex to an antisense oligonucleotide (ODN) carrier, and demonstrated that the antisense effect is drastically improved.[10] Another advantage to use SPG is that various chemical groups can be introduced to the side

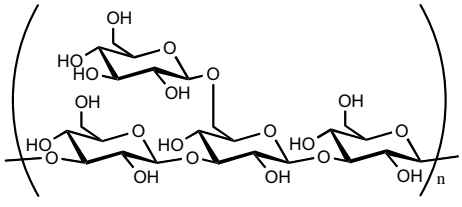

Figure 1. Chemical structure of schizophyllan.

Keywords: Polysaccharide/DNA complex; Schizopyllan; AFM.
☆This paper is series of polysaccharide–polynucleotide complex (36).
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chain and these do not interfere with the function of the bound antisense DNAs.[11] On the basis of these findings, we propose a new method to modify the end of double-stranded DNAs, as illustrated in Figure 2. Since poly(dA) forms the most stable complex with SPG, we have designed to attach a poly(dA) tail to the end of double-stranded DNAs. Using this tail, a functional group can be attached to the end of the DNA through the SPG/dA complex, followed by chemical modification of SPG. Since the complex shows nuclease resistance,[12] the terminal complex can play a role of the nuclease resistance cap similar to poxvirus genome, as well as a functional group carrier. Therefore, the proposed model can be a hybrid mimic of adenovirus and poxvirus. In this paper, we will construct the model complex depicted in Figure 2 and demonstrate this new concept by atomic force microscopy (AFM) and nuclease resistance assay.

SPG was kindly supplied by Taito Co. Ltd (Japan). The weight-average molecular weight and the number of repeating units were found to be 1.5×10^5 and 231, respectively. 2-Aminoethanol modified SPG (AESPG) was synthesized as described in a previous report.[13] The modification level (the molar ratio of cationic monomeric unit) was estimated to be 47.7 ± 2.2 mol% by nitrogen elemental analysis. We prepared a linear double-stranded DNA with poly(dA) tail at 5′ end, consisting of the transcription unit (CMV promoter, coding gene, RNA-stabilizing sequence) and SPG binding site (80 *mer*-dA bases) as schematically described in Figure 3.

A DNA fragment encoding an *Aequorea victoria*—enhanced green fluorescence protein (EGFP) was amplified by PCR, using pEGFP-C1 (Clonetech) as a template and 5′-GCATGCAGCGCACGAGGGAGCTT-3′ and 5′-GCATGCGGACTCTTGTTCCAAACTGGAAC-3′ sequences as the primers. The primers contain the *Sph* I restriction site, that is, GCATGC. The 2039-bp product in the PCR was incorporated into pGEM-T TA cloning vector (Promega). This plasmid was digested with *Sph* I and purified by agarose gel electrophoresis (denoted by fragment I). Two ODNs, whose sequences are 5′-CCGACAGCAAC-3′ and 5′-A_{80}GTTGCTGTCGGCATG-3′, were synthesized by Hokkaido System Science (Sapporo, Japan) and denoted by the adaptor ODN and the A80

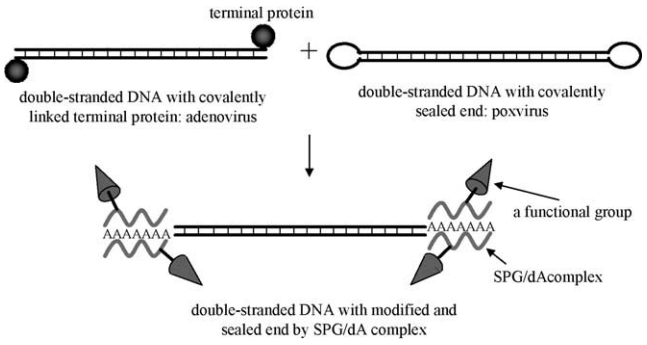

Figure 2. Proposed method in this paper to modify the end of double-stranded DNAs.

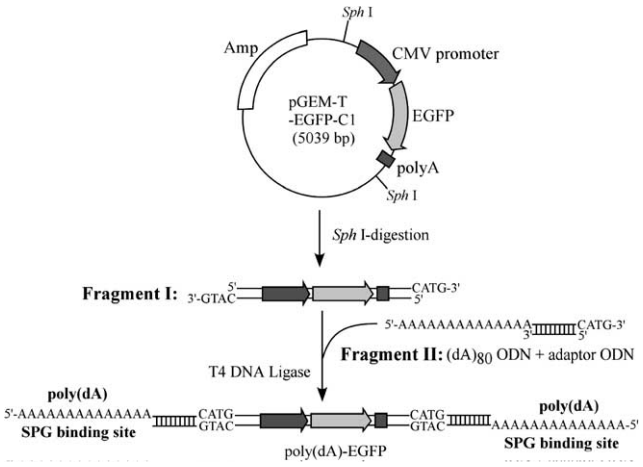

Figure 3. Preparation method of a poly(dA)-EGFP.

ODN, respectively. The 5′ end of the adaptor ODN was phosphorylated with ATP and T4 polynucleotide kinase (Promega). After the phosphorylation, the adaptor ODN and the A80 ODN were mixed and annealed to hybridize (to form fragment II, see Fig. 3). Fragment II has a poly(dA) tail at one end and the other end has a sticky sequence to fragment I. Ligation of fragments I and II was performed for 2 days at 4 °C with a 100-fold molar excess of fragment II and T4 DNA ligase (Promega). The final product was purified with oligo-dT magnet beads (Takara), followed by removing the residual fragment II with agarose gel electrophoresis. The presence of the poly(dA) tail at the both ends in the final product was verified by DNA sequencing and agarose gel electrophoresis for the fragments digested by *Sph* I. Hereinafter, we denote this final product as poly(dA)-EGFP. For a negative control, we used fragment I and denoted non-poly(dA)-EGFP.

Atomic force microscopic (AFM) images for the poly(dA)-EGFP complexed with SPG (or AESPG) were taken in tapping mode with a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA) equipped with NCH cantilevers (NANO WORLD, spring constant 42 N/m, resonant frequency 320 kHz). The samples were diluted to 2 ng/μL in DNA with a 2 mM $MgCl_2$ solution and cast onto freshly cleared mica. The mica was washed with distilled water to remove the buildup of excess salts on the surface. The details were described in elsewhere.[14,15] Nuclease resistance assay was carried out by BAL 31 nuclease, which possesses both 5′- to -3′ and 3′- to -5′ exonuclease activities. The complexed and naked DNAs (DNA 75 ng, and the SPG, AESPG weight ratio were 150) were dissolved in the reaction buffer (20 mM Tris–HCl pH 8.0, 600 mM NaCl, 12 mM $MgCl_2$, 12 mM $CaCl_2$, 1 mM EDTA). Subsequently, 0.75 U of BAL 31 nuclease was added to the solutions (final volume, 50 μL). The hydrolysis was monitored with a UV spectrometer.

Complexation between DNA and SPG (or AESPG) was accomplished by following the previous method.[9,13] Figure 4 compares the gel electrophoresis migration

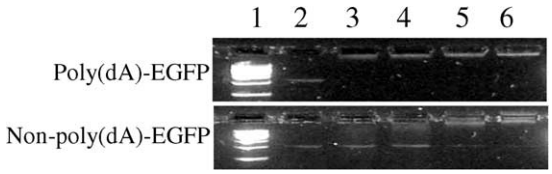

Figure 4. Electrophoretic retardation of DNA with AESPG on 1 wt% agarose gel electrophoresis. Lane 1 (1 kbp ladder), Lane 2 (1:0), Lane 3 (1:30), Lane 4 (1:60), Lane 5 (1:120), and Lane 6 (1:210), where the ratios in the parenthesis indicate the weight ratio of DNA to AESPG.

patterns between the mixtures of AESPG + poly(dA)-EGFP (upper) and of AESPG + nonpoly(dA)-EGFP (lower). The upper series of experiments show that the band retardation takes place when the AESPG weight ratio is increased to 1:30 or more. This retardation is considered to be an evidence for complexation between DNA and AESPG. However, this complexation does not necessarily mean the specific interaction between the poly(dA) tail and AESPG, but it can be just nonspecific ion-pair formation between positively charged AESPG and the negatively charged DNA. In contrast to the upper results, the mixtures of AESPG + non-poly(dA)-EGFP show that the band retardation starts when the ratio reaches 1:120. This difference clarifies the importance of the dA tail to induce the complexation between AESPG and poly(dA)-EGFP. In the lower panel, the band is retarded only when the extra amount of AESPG is added, enough to neutralize the negatively charged DNA. This feature can be explained by the fact that the poly-ion complex becomes immovable in the electric field when the ratio exceeds 1:120. In the upper panel, the retardation takes place at the ratio where the electric neutralization has not become dominant. Therefore, we can conclude that the retardation happening at the lower ratios (between 1:30 and 1:60) is the evidence for the complexation ascribable to the specific interaction between the poly(dA) tail and AESPG.

We found that there is no retardation observed (or difficult to observe) for the unmodified SPG + poly(dA)-EGFP mixture, although the dA tail/SPG complex is formed. This difficulty may be explained as following. SPG is electrically neutral and its molecular weight is considerably smaller than that of EGFP DNA (150 kDa for SPG and 1400 kDa for EGFP DNA, respectively). Therefore, even though the SPG/poly-(dA)-EGFP complex is formed, there should not be the appreciable difference in the molecular weights between the complexed poly(dA)-EGFP and naked one.

Figure 5 presents typical AFM images for the naked poly(dA)-EGFP (a), SPG/poly(dA)-EGFP complex (b), and AESPG/poly(dA)-EGFP complex (c), and Table 1 summarizes the average height obtained from the images, comparing the middle body and the tip end. The naked poly(dA)-EGFP shows curved-rod images and the height of the middle and tip are 0.40 and 0.45 nm, respectively. Essentially, no difference is observed between them. On the other hand, some of the SPG/poly(dA)-EGFP and AESPG/poly(dA)-EGFP complex images show that the DNA tip is covered by

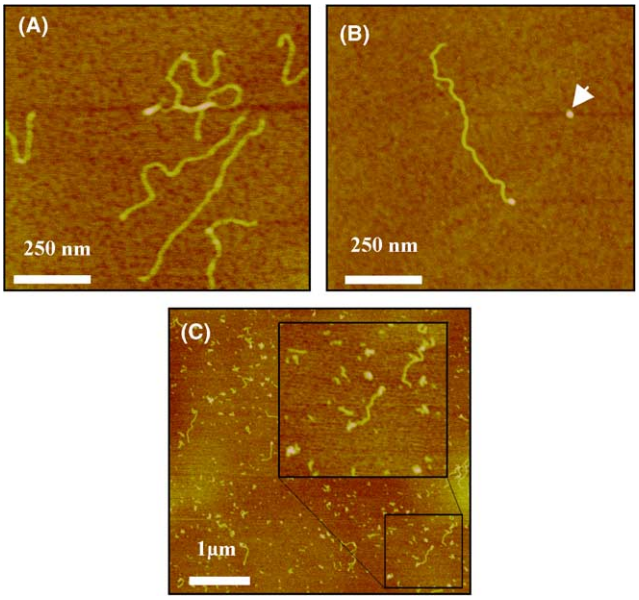

Figure 5. Comparison of AFM images among the naked poly(dA)-EGFP (a), SPG/poly(dA)-EGFP complex (b), and AESPG/poly(dA)-EGFP (c).

Table 1. Average heights of poly(dA)-EGFP and its complexes

	Height of DNA (middle) (nm)	Height of DNA (tips) (nm)	Height of free SPG or AESPG (nm)
Poly(dA)-EGFP[a]	0.40 ± 0.07	0.45 ± 0.10	—
SPG complex[b]	0.53 ± 0.07	1.65 ± 0.84	1.74 ± 0.82
AESPG complex[c]	0.42 ± 0.09	1.00 ± 0.31	0.99 ± 0.24

[a] Average of 20 DNA images.
[b] Average of 15 complex images.
[c] Average of 20 complex images.

a characteristic circular dot. Furthermore, isolated circular dots are observed (indicated by the arrow). The circular dots presumably represent SPG itself. This seems to contradict previous observations that renatured SPG showed a rod-like architecture.[16,17] However, sample preparation is totally different between the present work and the previous ones. In the previous works, they were just cast from an ultra-dilute solution of SPG onto the surface, any cations were not added to the diluting solution, and the sample was not washed with distilled water. These differences should create the difference in the image. In fact, when we treated the renatured SPG with the same manner with the complex, we observed the circular dots (data not shown). In our experiments, almost all of the renatured SPG were washed away with distilled water, and the remaining SPG on the mica should be observed as circular dots, probably because of compaction of SPG chains due to interaction between the hydroxyl groups of SPG and Mg^{2+}. Table 1 shows that the height at the tips are always taller than that of the middle part, confirming that SPG or AESPG binds to the tips of poly(dA)-EGFP.

Figure 5c shows the magnification of the AESPG/poly (dA)-EGFP complex. We found that AESPG always

binds to both of the DNA termini. In some case, depending on the washing conditions, the circular dot on the DNA tip was scarcely observed, probably because hard washing remove the bound AESPG. There was a very rare case that SPG attached to the middle of the DNA. We originally presumed that, because of the cationic nature, AESPG can bind the DNA with a nonspecific manner and there should have been no difference observed between poly(dA)-EGFP and non-poly(dA)-EGFP. However, our expectation was obviously wrong and AESPG had a higher affinity to bind the poly(dA) tail than the double-stranded parts. This conclusion is consistently supported by the gel electrophoresis and AFM results. When we carried out AFM for the SPG/poly(dA)-EGFP complex, the majority of the images are those in which one tip had the circular dots and the another tip had not such dots (see panel b). From the height observation, the tip without dots should correspond to the naked dA tail. Since the tail part consists of the fragment II, both ends are exactly identical. Therefore, we presume, that the naked dA tail may be caused by washed SPG from the complex, and the difference between the SPG and AESPG images of the complex can be ascribed to the difference in the binding affinity. Yazaki et al.[18] observed an electron microscopic image of adenovirus genome and found that it shows a dumbbell-like architecture owing to the terminal proteins attaching to the end of the linear double-stranded DNA. It should be emphasized than the image of AESPG/poly(dA)-EGFP (and SPG/poly(dA)-EGFP) complexes resembles that of adenovirus genome observed by Yazaki et al.

The most serious weak point of linear DNAs is to easily be degraded by exonuclease. The terminal protein of adenovirus should protect the end from the exonuclease degradation as well as works in the replication of the genome. Some viruses such as poxvirus have a covalently sealed end with the DNA itself (see Fig. 2), which is expected to protect the genome. The same strategy has been taken to protect the vector DNA from nuclease.[4–6,19]

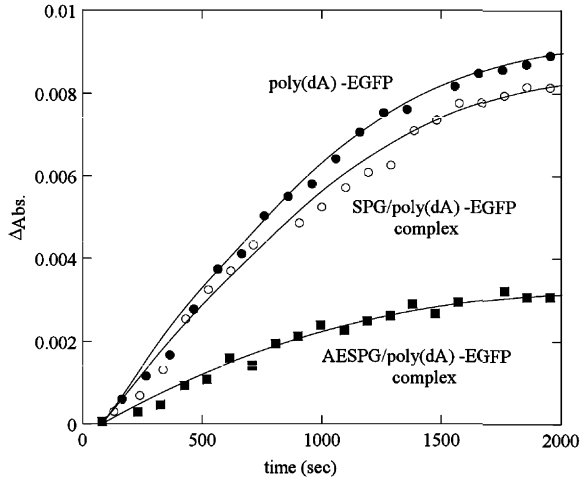

Figure 6. Time–course of the UV absorbance to show the stability of the complexed poly(dA)-EGFP with SPG or AESPG.

A question is whether the complexed dA tail can induce nuclease resistance. Figure 6 compares the time–course of the UV absorbance at 260 nm, where the increment of the absorbance is caused by nucleobases liberation from the staked double-stranded DNA.[20] Unmodified SPG complex shows a slightly slow degradation-rate than naked poly(dA)-EGFP. On the other hand, the AESPG complex showed excellent resistance. This feature can be related to the fact that the AESPG complex is much more stable the SPG one.

To sum up the report, as Kamiya et al.[4] pointed out, linear DNA vectors show a higher expression than the corresponding circular plasmid DNA, therefore, our proposed method may open a new possibility to improve the transfection and expression of linear DNA vectors. We are now in the process to evaluate the performance for AESPG/poly(dA)-EGFP and other related complexes.

Acknowledgements

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