Journal of Inclusion Phenomena and Macrocyclic Chemistry **41:** 173–178, 2001.

Novel DNA-Polysaccharide Triple Helixes and Their Application to a Gene Carrier

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Key words: complex, gene carrier, polynucleotides, polysaccharide, schizophyllan, triple helix

Abstract

Schizophyllan is a polysaccharide that belongs to the β-(1 $\rightarrow$ 3)-glucan family and has been known to activate the immunity system. This paper reviews our recent finding that schizophyllan can form a macromolecular complex with single-stranded polynucleotides and this complex is applicable to a new class of gene carriers.

Introduction

Schizophyllan [1, 2] and lentinan [3] are produced as a cell-wall polysaccharide by some funguses and have been known to cure gynecological cancers [4]. So far, this biological activity is explained by the fact that these glucans can activate the immunity system by promoting secretion of interleukins [5]. However, as shown by the chemical structure presented by Figure 1, schizophyllan and lentinan consist of only glucoses and no functional group and it seems that these polysaccharides have no way to interact with biomolecules.

Norisuye *et al.* [6, 7] extensively studied the dilute solution properties of schizophyllan and confirmed that it dissolves in water as a triple helix and in DMSO as a single chain (s-SPG). Furthermore, when water is added to the DMSO solution, s-SPG collapses owing to the hydrophobic interaction and forms both intra- and intermolecular hydrogen bonds (renaturing process) [8]. Although the renatured product is not the same as the original rod like molecules, the local structure is expected to retrieve the triple helix [9]. Sakurai and Shinkai found that when some polynucleotide coexists in the renaturing process, the nucleotide and s-SPG form a novel macromolecular complex [10]. This paper reviews our recent results on this novel polysaccharide/polynucleotide interactions [10–14].

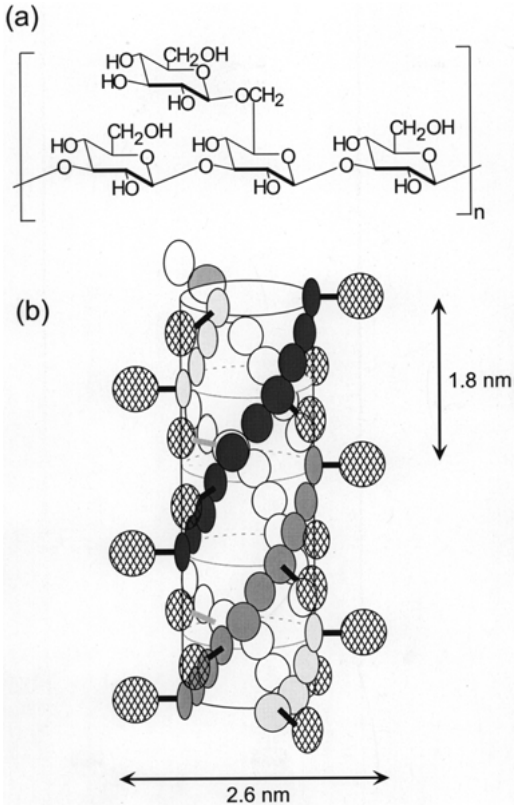

Figure 1. Repeating unit of schizophyllan (a) and its representative model of the triple-helix (b). In the panel (b), the plain circles represent the main chain glucose residues and the meshed ones, the side chains. Lentinan has two β-(1 $\rightarrow$ 6) glucose side chains out of the five β-(1 $\rightarrow$ 3) glucose main chain.

Stacking of base molecules upon complexation [12]

Figure 2 presents the UV and circular dichroism (CD) spectra for the poly(C) system, where poly(C) is poly(cytidine monophosphate) and the complex is denoted as poly(C)/s-SPG. The complexation decreases the absorbance of cytosine by 12% (hypochromism) and moves λ_{max} slightly

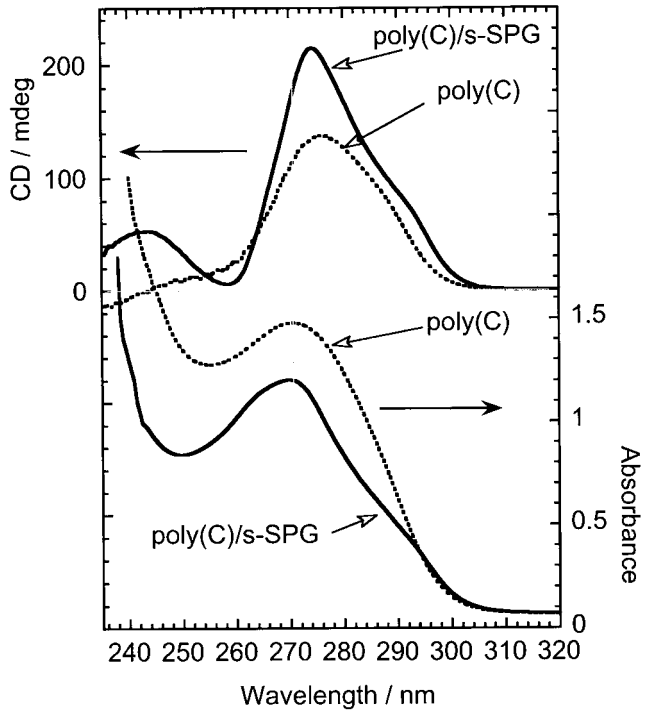

Figure 2. Comparison of the UV and CD spectra between poly(C) and its complex measured at 10 °C for $C_{poly(C)} = 7.16 \times 10^{-3}$ g dL^{-1} (2.22×10^{-4} M) with a 1 cm cell for both the CD and UV.

to lower wavelength (blue shift) in the UV spectra. Furthermore, it increases the CD intensity of the 280 nm positive. According to previous work [15, 16], all these changes take place when stacking of the base molecules is enhanced. Therefore, we can conclude that stacking between the cytosine bases is enhanced upon the complexation.

Base molecule specificity [13]

Table 1 summarizes which homo-polynucleotide can induce the complexation comparing with their conformations. None of poly(G), poly(U), poly(I), poly(dG), and poly(dC) shows complexation; however, we found that poly(C), poly(A), poly(dA) and poly(dT) can form a complex with s-SPG. According to the previous work [17] the guanines in poly(G) and poly(dG) form a tertamer, the uraciles and cytosines in poly(U) and poly(dC) form a dimer, and the inosines in poly(I) form a tetramer or trimer. For all the cases the hydrogen-bonding sites in the bases are used to form the intramolecular interaction. On the other hand, poly(C), poly(A), poly(dA), and poly(dT) do not form such an intramolecular aggression, therefore, their hydrogen-bonding sites are unoccupied. There is clear correspondence between the ability of the interaction and the presence of the free hydrogen-bonding sites. Namely, only when the hydrogen bonding-site is available, the polynucleotide can interact with s-SPG. This correspondence evidences that the hydrogen-bonding interactions are essential to induce the interaction. This leads to the conclusion leads that s-SPG and polynucleotides form a macromolecular complex.

Table 1. Nucleotide specificity in the complex formation in neutral and non-salt aqueous solution

		Complex formation	Conformation
RNA	poly (C)	Yes	Single chain
	poly(A)	Yes	Single chain
	poly(U)	No 7	Intramolecular H-bond (hairpin like)
	poly(G)	No	4G wire (intramolecular H-bond)
	poly (I)	No 7	Intramolecular H-bond
DNA	poly(dC)	No	Intramolecular H-bond
	poly(dA)	Yes	Single chain
	poly(dT)	Yes	Single chain
	poly(dG)	No	4G wire (intramolecular H-bond)

Temperature and composition dependence of circular dichroism of the complex [14]

Figure 3 compares the temperature dependence of $[\theta]_{max}$ between polynucleotides and their complexes, where $[\theta]_{max}$ is the CD intensity as the top of positive band. As seen in the figure, there is an abrupt decrease in $[\theta]_{max}$ around 32 and 54 °C for poly(A)/s-SPG and poly(C)/s-SPG, respectively. Furthermore, above the each critical temperature, the mixture's $[\theta]_{max}$ merges in the same plots of the corresponding polynucleotide itself. These features show that s-SPG/polynucleotide complexes are formed only at lower temperatures and they dissociate upon heating. The abrupt decrease is associated with the autoaccelerative dissociation of the complexes. It is interesting that this abrupt decrease is similar with the well-known melting behavior of double-helix polynucleotides. The poly(C)/s-SPG complex "melts" at higher temperature than that of the poly(A)/s-SPG complex, which is similar to the fact that the poly(C)/poly(G) complex melts at higher temperature (ca. 110 °C) than that of the poly(A)/poly(U) complex (ca. 65 °C). The difference in the DNA helix melting temperature can be ascribed to the difference in the number of the hydrogen bonds (cytosine has 3 hydrogen-bonding sites whereas adenosine has the 2 sites). The same explanation should be applicable to the difference in the melting temperature of the s-SPG/polynucleotide complexes.

Figure 4 plots the relative intensities of CD against V_w for both poly(A) and poly(C) systems. Here, V_w is the water volume fraction in a water/DMSO solution and the relative intensity is defined as the normalized value of $[\theta]_{\lambda_{max}}$ by that of poly(A) or poly(C) at $V_w = 1$. In the low V_w region, the individual polynucleotide, i.e., poly(A) or poly(C), exhibits the same values as expected for each mono-nucleotide. At $V_w = 0.5$ for poly(A) and $V_w = 0.6$ for poly(C), the intensity begins to increase. These increments indicate that polarity of the solvent becomes significant enough to induce the hydrophilic interaction between the corresponding bases. With increasing V_w, the intensity increases essentially linearly with V_w. On the other hand, if s-SPG is added, it presents completely different behavior from those of each individual

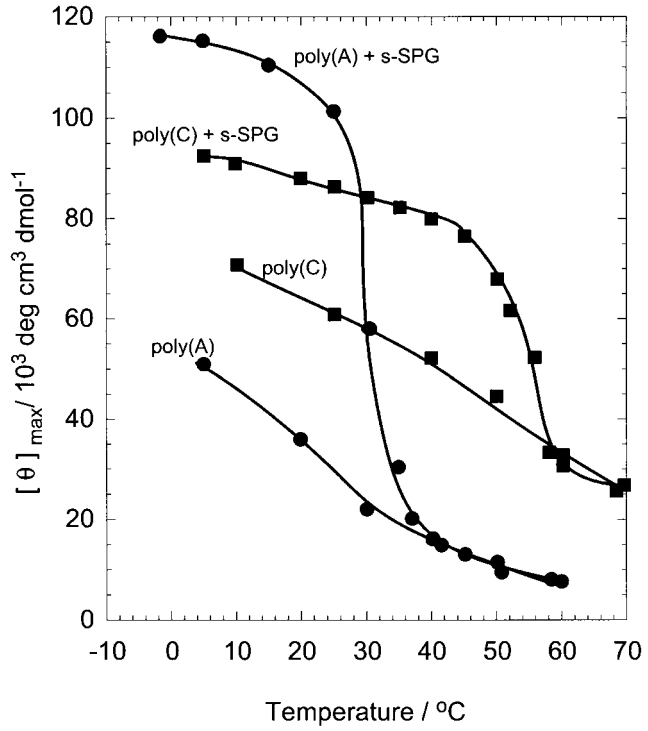

Figure 3. Temperature dependence of $[\theta]_{max}$ for the polynucleotides and their mixtures with s-SPG.

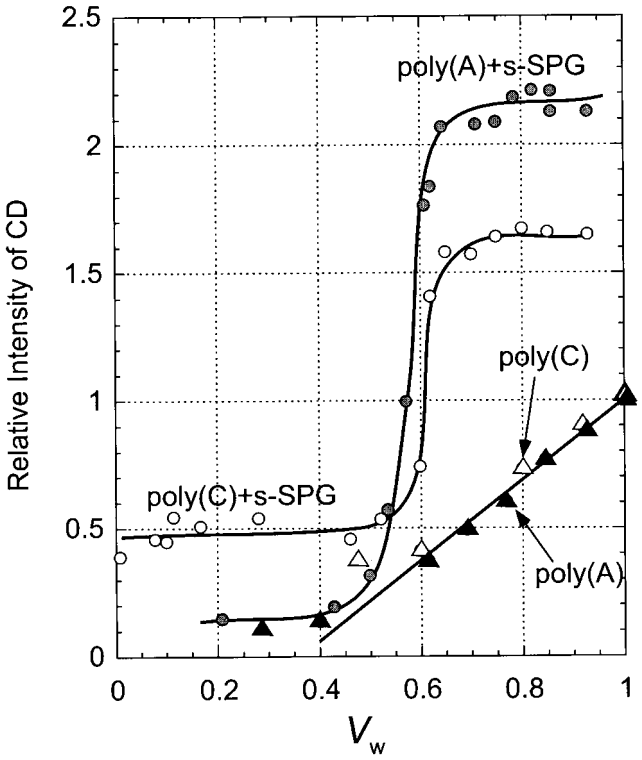

Figure 4. Composition dependence of the relative intensity of CD for poly(C), poly(C) + s-SPG, poly(A), and poly(A) + s-SPG.

polynucleotide. In the low V_w region (ca. 0–0.45) the CD intensity stays at the same value as that of the mononucleotide. At $V_w = 0.58$ for poly(A) + s-SPG and $V_w = 0.6$ for poly(C) + s-SPG, the intensities drastically increase and they level off at $V_w > 0.7$. The saturation of the CD intensity indicates that the complex formation is completed and most bases are stacking in $V_w > 0.7$. This composition dependence clearly shows importance of hydrophobic interaction to trigger the cooperative formation of the complex.

Stoichiometry and molecular modeling [14]

Although the data are not shown here, further experiments were carried out to Determine the stoichiometry of the poly(A), poly(dA) and poly(C) systems [13, 14]. The results indicate that two SPG repeating units bind with three base units, namely, 8 glucose residues vs. 3 nucleic bases. By the way, we already know that schizophyllan uses the C-2's OH group of the main-chain glucose to form hydrogen-bonds (see inset A in Figure 5) and that the side-chain glucose only provides solubility, thus it is not involved in the helix formation. Therefore, we can presume that the s-SPG side chain in the complex behaves in the same manner as that in the schizophyllan triple helix. Thus, we suppose that (1) 6 glucose residues (8 minus 2 side glucoses) are interacting with 3 bases as shown in the inset B in Figure 5, (2) a triple helix is formed in the complex and (3) the same OH group is involved in the hydrogen-bonding in the complex. Combination of the stoichiometric result and the above assumptions leads to the most plausible model as depicted in Figure 5. This model means that when the renaturing process is carried out in the presence of polynucleotides, two s-SPG chains and one polynucleotide chain retrieve the triple helix.

Based on the above model, we estimated the likely stable structure by means of molecular mechanics. The results are presented in Figure 6 as the overall CPK model and as a stick model magnifying the hydrogen bonding and stacking part. The poly(C) chain fits the groove, left by the 3rd SPG chain and the calculation indicates that there is no obstruction in steric hindrance. This is reasonably understood because of the similarity in the helix parameters between poly(C) and schizophyllan. Schizophyllan and poly(C) form a right handed 61 triple helix with a 17.4 Å pitch and a right-handed 61 helix with a 18.6 Å pitch, respectively. The model predicts that the amino group attached to N-4 donates its proton to O atom of C-2 in the adjacent glucose and the puckering of the ribose is 3′ end (i.e., the A form). The base distance is 3.0 Å, which agrees with the results of the X-ray crystallography for the poly(C)/s-SPG complex [14].

Application to a new gene carrier [14]

DNA protection from ribonucleases is one of the key issues in designing antisense DNA carries of gene therapy [18]. Most investigations have been focused on the complexes made from DNA and polycations such as polylysine and polyethylenimine [19]. We examined how poly(C) in the complex resists the hydrolysis by RNase A. Although the data are not shown here, the hydrolysis rate is dramatically reduced upon the complexation. The maximum velocities, evaluated form the Linweaver-Burk plot, were

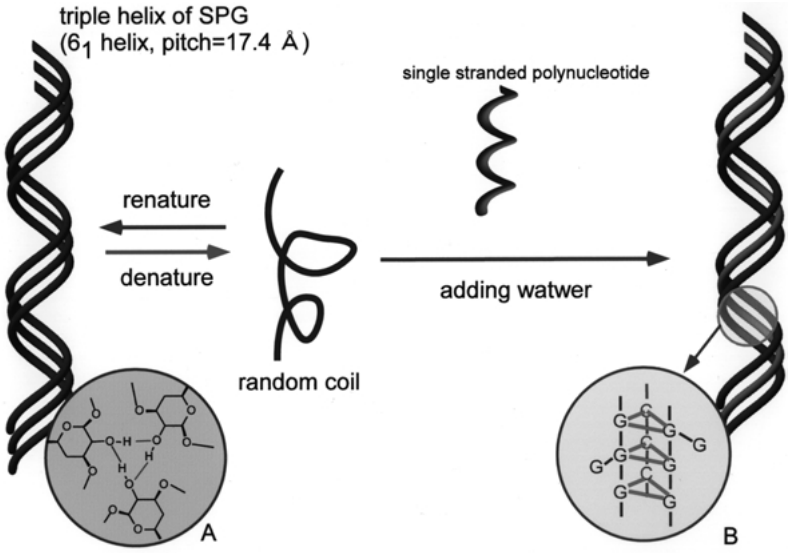

Figure 5. Schematic illustration of the complex formation form polynucleotide and s-SPG. Blue and red lines represent the s-SPG and polynucleotide molecules, respectively. In the inset, the capital letters of G and C represent the glucose and base, and the blue lines shows the hydrogen bonds, respectively.

6.6 and 1.2 $\times$ 10^{-7} M sec^{-1} for the control and the complex, respectively. Thus, we can conclude that the s-SPG/poly(C) complex can resist the hydrolysis much better than polycation/polynucleotide complexes.

We examined how the complex protects antisense DNA and enhances inhibition of translation in a biological system. Figure 7 compares inhibition of GFP (green fluorescence protein) translation between an antisense DNA itself and a complex of the antisense DNA and s-SPG. The results clearly show that expression of GFP is more suppressed in antisense DNA/s-SPG than in antisense itself. Therefore, we can conclude that the complex successfully takes the antisense DNA to the target RNA and delivers it to the RNA.

Experimental methods

Taito Co. in Japan kindly supplied the triple helix of schizophyllan sample. The molecular weight and the number of repeating units were found to be 1.5 $\times$ 10^5 and 231, respectively. Mixtures of a polynucleotide and s-SPG were prepared by adding a s-SPG solution in DMSO to a polynucleotide solution in water and the apparent pH of the DMSO/water solution was found to be 8.0–8.5. The CD spectra in the 230–320 nm region were measured in the temperature range of 5–70 °C on a Jasco J-720WI spectropolarimeter. Absorbance change at 270 nm was measured at 37 °C with a Jasco V-570 spectrometer to determine the hydrolysis velocity. When we converted the absorbance change with time to the velocity, we used the following extinction coefficients in the DMSO/water solution at 37 °: 7.3 $\times$ 10^3 M^{-1} cm^{-1} for cytidine-3′-monophosphate, 6.65 $\times$ 10^3 M^{-1} cm^{-1} for poly(C) and 5.68 $\times$ 10^3 M^{-1} cm^{-1} for the s-SPG/poly(C). The molecular mechanics calculation was done with the *Discover 3* program using the Amber force field. The *in-vitro* transcription/translation assay was carried out using *E. coli* T7 S30 Extract system for circular DNA (Promega) and a GFP expression vector; pQBI63 (Takara). The antisense DNA sequence was CTTTAAGAAGGAGATATACAT-$(A)_{40}$. The $(A)_{40}$ tail is attached to increase affinity to bind with s-SPG and the sequence is designed to target the ribosome binding site (1043-1064 region of GFP). CD and gel-electrophoresis confirmed complex formation between this antisense DNA and s-SPG.

Concluding remarks

This report clarifies the molecular mechanism and structure of a novel polysaccharide/polynucleotide complex and shows potentiality to apply the complex to a new gene carrier. Our work is the first clear finding that a polysaccharide can specifically interact with polynucleotides, except for some oligosaccharides such as calicheamicines. We expect that the present findings will provide an important clue to clarify saccharide-polynucleotide interactions that frequently play a critical role in biological systems. Ability of s-SPG to bind with single chains of polynucleotides may provide a new explanation for the antitumor activity of the β-(1 $\rightarrow$ 3)-glucan family. Tumor cells tend to absorb larger molecules with endcytosis than normal cells [20]. Therefore, s-SPG in blood has more chance to transfer to tumor cells than to normal cells. When s-SPG enters the inside of the cell, it can bind with the single-chain of polynucleotides such as a poly(A) tail of mRNA. Once this happens, the central dogma is interfered and presumably the tumor cell activity is reduced.

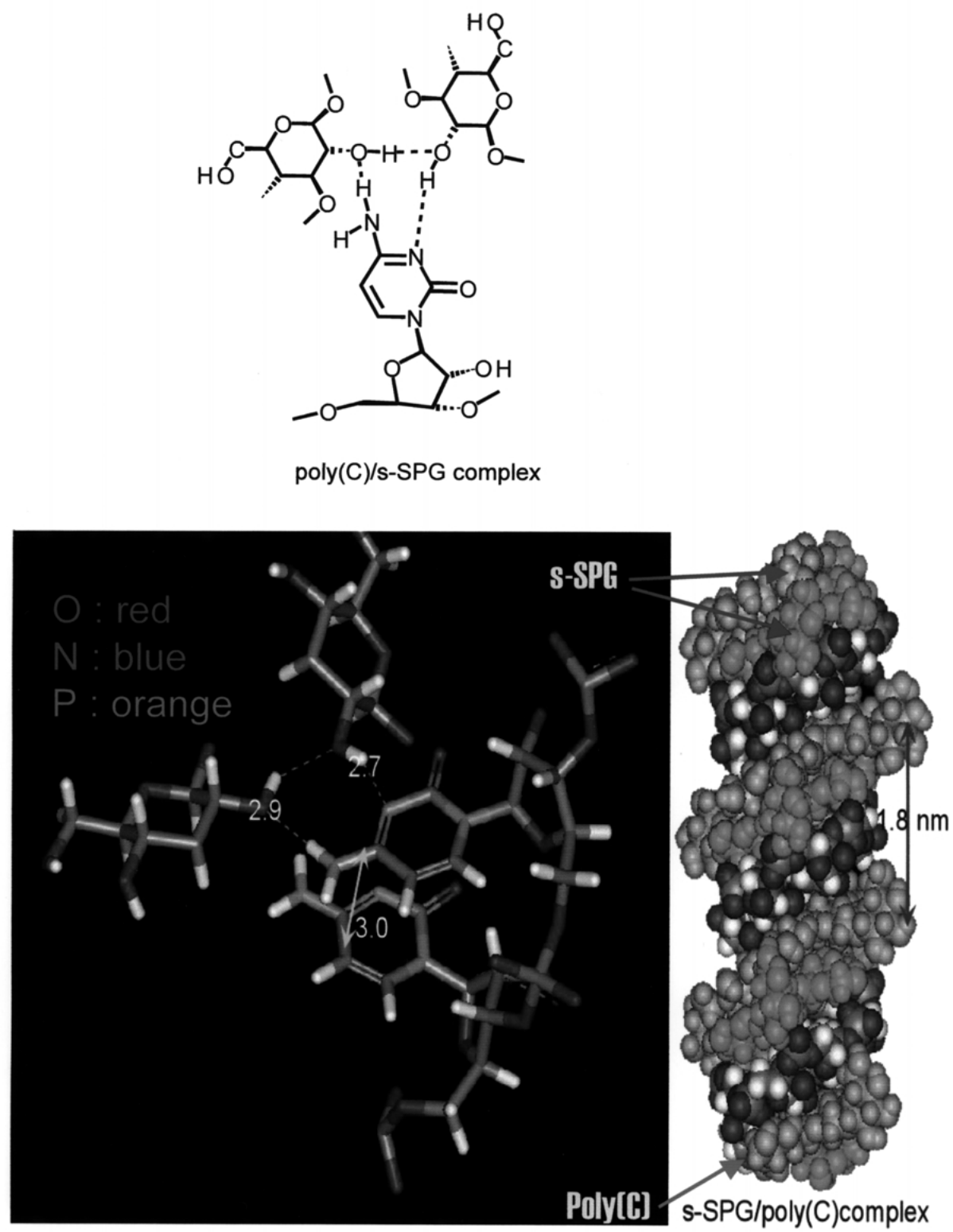

Figure 6. Molecular model of the complex calculated by means of molecular mechanics. The CPK model in the right hand side shows an overall structure of the triple helix made from two s-SPG chains (pink and green) and poly(C). A magnified structure in the right hand side shows the hydrogen bonding site and the base stacking. In both the figures, red, white, orange, and blue colors represent O, H, P, and N atoms, respectively. The numbers in the figure shows distance in Å scale.

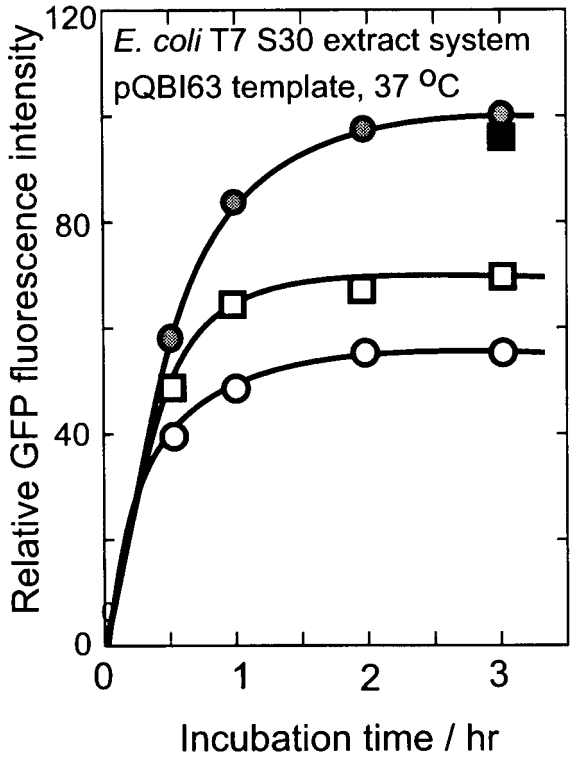

Figure 7. Comparison of GFP expression between the antisense DNA (antisense + template) and its complex with s-SPG (antisense/s-SPG + template). Time course for GFP increment expressed by the template pQBI 63 vector is also plotted as a reference (template only).The GFP fluorescence intensity excited by 460 nm light was measured at 506 nm and all values obtained for the reference system after 3 hr. To examine how s-SPG affects this expression system, fluorescence was measured for a mixture of the template DNA and an extra amount of s-SPG (plotted as a square symbol).

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