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Synthesis of long Poly(dG)·Poly(dC) DNA using enzymatic reaction†

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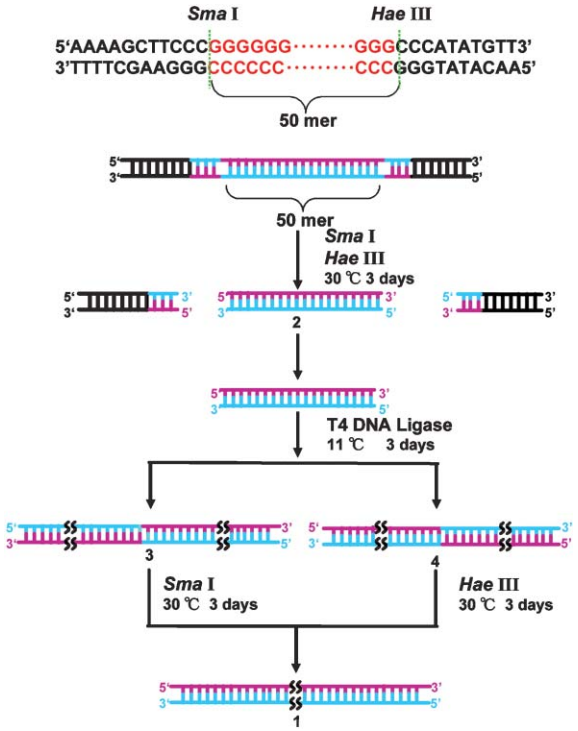
Non-defect Poly(dG)·Poly(dC) of 500 bp (170 nm) has been synthesized by using enzymatic reactions and was characterized by its UV spectrum, showing that conjugated π-electrons between base pairs are spread over the DNA molecule suggesting the absence of structural defects.

Guanine rich strands exist at the end of a chromosome and is denoted a telomeric structure. It is suggested that this structure is associated with cellular aging, since this structure controls the number of cell divisions. Up to now, in order to investigate telomeric structures, DNA with a G- and C-rich strand[1–4] or a repeated sequence of ATTTT[5,6] have been synthesized up to 1000 bp with telomerase[7,8] the enzyme which elongates the telomere sequence in DNA. However, homopolymer double stranded DNA has not been synthesized with more than 1000 bp. Particularly, it is difficult to synthesize homopolymer Poly(dG) which consists of only guanine in the same strand of more than 100 bp, since a guanine higher-order structure (guanine quadruplex) is formed in the synthesis.

We already succeeded in synthesizing Poly(dA)·Poly(dT) of a length of more than 1000 base pairs with no structural defects using a multi-step enzymatic reaction.[9] We then applied the same technique to synthesize Poly(dG)·Poly(dC). In this case, however, we failed to synthesize long-chain Poly(dG)·Poly(dC) because single chain Poly(dG) formed a higher-order structure between and in the molecules.[1–4] The guanine quadruplex is more stable than the guanine–cytosine pair, T_m (melting temperature) of the guanine quadruplex[10] being much higher than that of the guanine–cytosine pair. On the other hand, conventional methods can produce double-stranded DNA, though the DNA always has structural defects due to the incomplete hybridization of single strands. We therefore tried to find another synthesis approach to avoid the formation of a higher-order structure of Poly(dG), using not a single strand but a double strand as a starting material.

The synthesis procedure is shown in Scheme 1. We started with DNA of 70 base pairs (base sequence 5′AAAAGCTT-CCCGG···GGCCATATGTT3′). In the center of the DNA, there is a sequence of 50 successive bases of guanine, and the double helix structure is already formed with the complementary chain of this DNA in order to prevent the formation of the higher-order guanine structural unit. We first applied the restriction enzyme, *Sma* I (Toyobo) and *Hae* III (Toyobo), to the substrate DNA to cut it in to three pieces: two end parts and Poly(dG)·Poly(dC) **2** of 50 base units. *Sma* I is an enzyme that recognizes the base sequence 5′-CCCGGG-3′/3′-GGGCCC-5′ and selectively cuts the guanine–cytosine bond. *Hae* III is an enzyme that recognizes the base sequence 5′-GGCC-3′/3′-CCGG-5′ and selectively cuts the guanine–cytosine bond. We allowed reaction at 30 °C for 3 days after adding (5 μl) of TA buffer (× 10) ,[11] 3 μl of *Sma* I (12 U μl^{-1}), and 3 μl of *Hae* III (10 U μl^{-1}) to 39 μl of substrate DNA solution (109.5 μM). In order to keep the enzyme activated during the reaction, we added 3 μl of *Sma* I (12 U μl^{-1}) and 3 μl of *Hae* III (10 U μl^{-1}) every 24 h. After the reaction we separated, purified and collected Poly(dG)·Poly(dC) **2** using HPLC.[12]

† Electronic supplementary information (ESI) available: STM images. See http://www.rsc.org/suppdata/cc/b4/b410753e/



Scheme 1 Synthesis of long-chain Poly(dG)·Poly(dC).

We then applied the DNA joining enzyme, T4 DNA Ligase, to the purified 50-base Poly(dG)·Poly(dC) **2** to activate the ligation and synthesized a long-chain DNA.

Next, the 50-base Poly(dG)·Poly(dC) **2** was dissolved in 34 μl of ultrapure water. We added 7 μl of TA buffer (× 10), 5 μl of ATP (adenosine 5′-triphosphate) (1 mM) and 2 μl of T4 DNA ligase (40 U μl^{-1}) to the DNA solution and allowed reaction at 11 °C for 3 days. In order to prevent the deactivation of the enzyme during the reacting process, we added 2 μl of T4 DNA ligase (40 U μl^{-1}) every 24 h. This reaction produces guanine–guanine (cytosine–cytosine) **1** homo-bonding as well as cytosine–guanine **3** (guanine–cytosine **4**) hetero-bonding. Again, we applied *Sma* I and *Hae* III to cut the guanine–cytosine and cytosine–guanine bonds so that only the homopolymer Poly(dG)·Poly(dC) **1** would remain. For this reaction we added 3 μl of *Sma* I (12 U μl^{-1}) and 3 μl of *Hae* III (10 U μl^{-1}) and left them at 30 °C for 3 days. Deactivation of the enzyme was prevented by adding 3 μl of *Sma* I (12 U μl^{-1}) and 3 μl of *Hae* III (10 U μl^{-1}) every 24 h during the reaction. Finally, we applied S1 nuclease to the Poly(dG)·Poly(dC) **1** to remove the single chain species expected to exist in the DNA. S1 nuclease recognizes nick and single-chain regions (structural defects) in DNA and selectively cuts more than 90% of these efficiently. For the reaction we added 3 μl of S1 nuclease (160 U μl^{-1}) and 8 μl of S1 nuclease reaction buffer (× 10) to 70 μl of the reaction solution and allowed reaction at 37 °C for 15 min. We then purified and collected the DNA by phenol extraction and ethanol precipitation. We also separated DNA by polyacrylamide gel electrophoresis and

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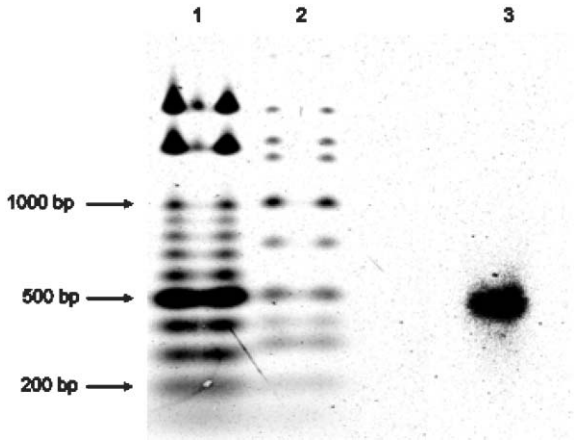

Fig. 1 Agarose gel electrophoresis of synthesized Poly(dG)·Poly(dC): 2.0% agarose gel was used. Lane 1 is 100 base pair (bp) Ladder (Toyobo co., Ltd) and lane 2 is Hi–Lo DNA Marker (Bionexus, Inc.). Lane 3 is the presently synthesized Poly(dG)·Poly(dC). The sample was run in TBE buffer (89 mM Tris-base–89 mM boric acid–2 mM EDTA (pH = 8.0)) with a voltage of 20 V applied between the anode and cathode for 18 h. The gel was stained by SYBR Green I stain (Molecular Probes).

excised gel of around 500 bp. Purification and collection of 500 bp Poly(dG)·Poly(dC) **1** was performed by using electroelution and ethanol precipitation.

We evaluated the length of synthesized Poly(dG)·Poly(dC) by electrophoresis (Fig. 1). One band was confirmed around 500 bp in lane **3** and thus 500 bp Poly(dG)·Poly(dC) was efficiently synthesized. We concluded that there were no nicks or structural defects in the synthesized 500 bp Poly(dG)·Poly(dC). On the other hand, since commercial Poly(dG)·Poly(dC) has structural defects at intervals of 50 to 100 bp, this DNA was degraded below 100 bp by S1 treatment.

The 500 bp DNA structure was investigated by circular dichroism measurements. The DNA concentration in 10 mM NaCl solution was about 60 μg ml^{-1}. As shown in Fig. 2, we found a positive peak around 260 nm and a negative peak around 240 nm in the CD spectrum. In general, the CD spectrum of DNA with right-handed helical structure (B-form) has a positive peak at 260 nm and a negative peak at 240 nm.[13,14] In addition, the CD spectrum of this DNA differed from that of the left-handed helical structure[15] and of the guanine quadruplex.[16] Furthermore, in STM observation (ESI†), we confirmed that this DNA forms a double strand. Accordingly, our DNA is of the B-form.

Finally, the UV spectrum of the synthesized 500 bp Poly-(dG)·Poly(dC) was measured (Fig. 3). The concentration of commercially available Poly(dG)·Poly(dC) and the synthesized 500 bp Poly(dG)·Poly(dC) in 10 mM NaCl solution were about 24 and 60 μg ml^{-1}, respectively. The UV spectrum of commercial Poly(dG)·Poly(dC), which contains structural defects, shows an adsorption maximum at 257 nm. On the other hand, for the synthesized Poly(dG)·Poly(dC), the adsorption maximum was observed at 265 nm *i.e.* shifted to longer wavelength. Since the adsorption maximum of general double strand DNA is observed around 260 nm in the UV-vis spectrum, this indicates that

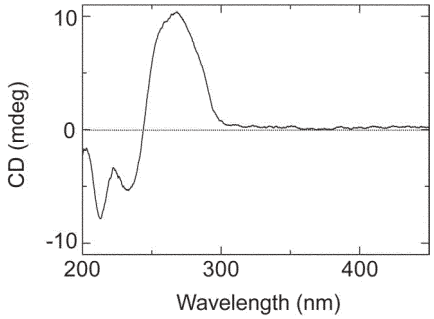

Fig. 2 Circular dichroism spectrum of synthesized 500 bp Poly(dG)·Poly(dC) in 10 mM NaCl solution. The concentration of DNA is about 60 μg ml^{-1}.

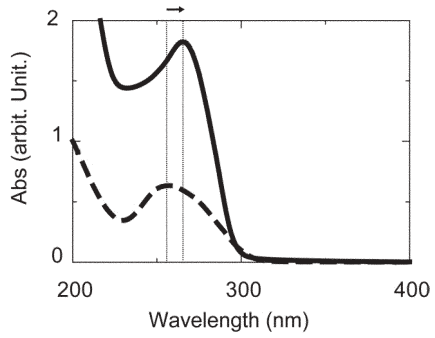

Fig. 3 UV-Vis spectrum of synthesized 500 bp Poly(dG)·Poly(dC) (solid line) and commercial Poly(dG)·Poly(dC), which contains structural defects (dashed line). In the measurement of UV spectra an Ultrospec 3300 pro (Amersham Bioscience) was used. The concentration of commercially available Poly(dG)·Poly(dC) and the synthesized 500 bp Poly(dG)·Poly(dC) were about 24 and 60 μg ml^{-1}, respectively, and the measurements were performed in 10 mM NaCl solution.

π-electrons between base pairs are spread over the DNA molecule owing to the absence of structural defects. Furthermore, we used STM to observe the fine structure of the synthesized Poly(dG)·Poly(dC) (ESI†). The STM image shows that the synthesized DNA is linear and has no bending structure that is seen in single-chain parts of the commercial DNA. Therefore the synthesized DNA has a double helix structure with no defects.

In conclusion, we have succeeded in the synthesis of defect-free Poly(dG)·Poly(dC) of length up to 500 bp (170 nm) by use of enzymatic reactions. In future work we will evaluate the electronic properties of Poly(dG) ·Poly(dC) without structural defects synthesized in this study, and aim at applying it as a novel electrical material.

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11 TA buffer (×10): Tris-Acetate (33 mM, pH = 7.9), KOAc (66 mM), $MgOAc_2$ (10 mM), Dithiothreitol (0.5 mM).
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