

# Branched-Chain Polyamine Found in Hyperthermophiles Induces Unique Temperature-Dependent Structural Changes in Genome-Size DNA

Takashi Nishio,<sup>[a]</sup> Yuko Yoshikawa,<sup>[a]</sup> Wakao Fukuda,<sup>[b]</sup> Naoki Umezawa,<sup>[c]</sup> Tsunehiko Higuchi,<sup>[c]</sup> Shinsuke Fujiwara,<sup>[b]</sup> Tadayuki Imanaka,<sup>[d]</sup> and Kenichi Yoshikawa\*<sup>[a]</sup>

A pentavalent branched-chain polyamine,  $N^4$ -bis(aminopropyl)spermidine 3(3)(3)4, is a unique polycation found in the hyperthermophilic archaeon *Thermococcus kodakarensis*, which grows at temperatures between 60 and 100 °C. We studied the effects of this branched-chain polyamine on DNA structure at different temperatures up to 80 °C. Atomic force microscopic observation revealed that 3(3)(3)4 induces a mesh-like structure on a large DNA (166 kbp) at 24 °C. With an increase in

temperature, DNA molecules tend to unwind, and multiple nano-loops with a diameter of 10–50 nm are generated along the DNA strand at 80 °C. These results were compared to those obtained with linear-chain polyamines, homocaldopentamine 3334 and spermidine, the former of which is a structural isomer of 3(3)(3)4. These specific effects are expected to neatly concern with its role on high-temperature preference in hyperthermophiles.

## 1. Introduction

Polyamines are found in all living organisms, where they play important roles in many cellular processes including cell growth and proliferation.<sup>[1–3]</sup> The most commonly occurring natural polyamines are putrescine [2+], spermidine [3+] and spermine [4+], all of which have a linear chain structure. Linear analogues of these polyamines, such as norspermidine [3+], norspermine [4+], thermospermine [4+] and homocaldopentamine [5+], which differ with regard to the length and arrangement of CH<sub>2</sub> spacers and valency, are also found in various organisms.<sup>[4,5]</sup> It is known that polyamines induce DNA condensation/compaction because of their polycationic nature at physiological pH.<sup>[6]</sup> A number of *in vitro* studies have investigated the physicochemical mechanism of polyamine-induced DNA condensation/compaction.<sup>[7–25]</sup> These *in vitro* studies shed some light on the manner of packing of DNA in living systems.<sup>[26,27]</sup>

Recently, it was reported that a hyperthermophilic archaeon microorganism, *Thermococcus kodakarensis*, synthesizes a unique branched-chain polyamine  $N^4$ -bis(aminopropyl)spermidine 3(3)(3)4 that has not been found in other microorganisms.<sup>[28,29]</sup> *T. kodakarensis* grows at temperatures between 60 and 100 °C, with an optimum growth temperature of 85 °C.<sup>[30]</sup> Interestingly, synthesis of the branched-chain polyamine 3(3)(3)4 increases with an increase in the growth temperature.<sup>[28]</sup> Therefore, this branched-chain polyamine may be a key molecule for survival in high-temperature environments.<sup>[28]</sup>

We previously investigated the effects of branched- and linear-chain polyamines on the higher-order structure of genome-size DNA at room temperature by the use of fluorescence microscopy and atomic force microscopy (AFM).<sup>[31]</sup> We found that branched-chain polyamines tend to induce a meshwork assembly of randomly oriented DNA fibers, whereas linear-chain polyamines cause a parallel alignment between DNA segments.<sup>[31]</sup> Circular dichroism (CD) measurements revealed that branched-chain polyamines induce the A-like form in the secondary structure of DNA, while linear-chain polyamines have only a minimal effect.<sup>[31]</sup>

Here we extended this work by focusing on the temperature-dependence of the interactions of polyamines with DNA. We evaluated the temperature-dependent effect of a pentavalent branched-chain polyamine, 3(3)(3)4, on DNA structure. The results were compared to those obtained with linear-chain polyamines, spermidine SPD and homocaldopentamine 3334 which is a structural isomer of 3(3)(3)4. Their chemical structures are shown in Scheme 1. We used a genome-size DNA, T4 GT7 DNA (166 kbp), to monitor the change in conformation by AFM observation. It has been shown that the size or length of DNA molecules is a critical determinant governing their overall morphological features.<sup>[32–36]</sup> Teif reported that long enough DNA molecules can collapse into compact globules with various shapes such as a toroid or rod,

[a] T. Nishio, Dr. Y. Yoshikawa, Prof. K. Yoshikawa  
Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe 610-0394, (Japan)

E-mail: keyoshik@mail.doshisha.ac.jp

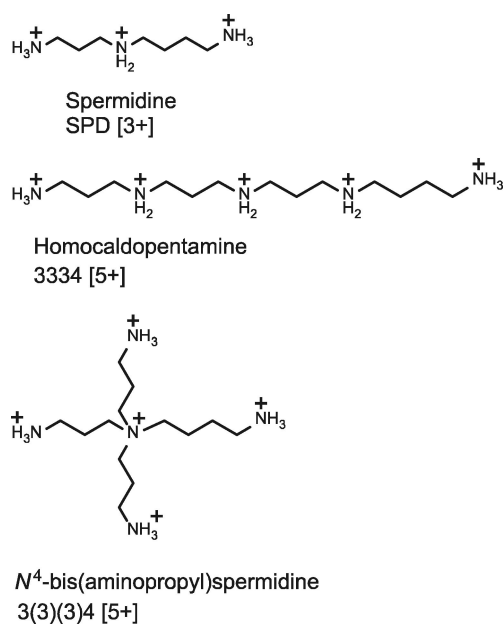
[b] Dr. W. Fukuda, Prof. S. Fujiwara  
School of Science and Technology, Kwansai-gakuin University, Sanda 669-1337, (Japan)

[c] Dr. N. Umezawa, Prof. T. Higuchi  
Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya 467-8603, (Japan)

[d] Prof. T. Imanaka  
Research Organization of Science and Technology, Ritsumeikan University, Kusatsu 525-8577, (Japan)

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cphc.201800396>

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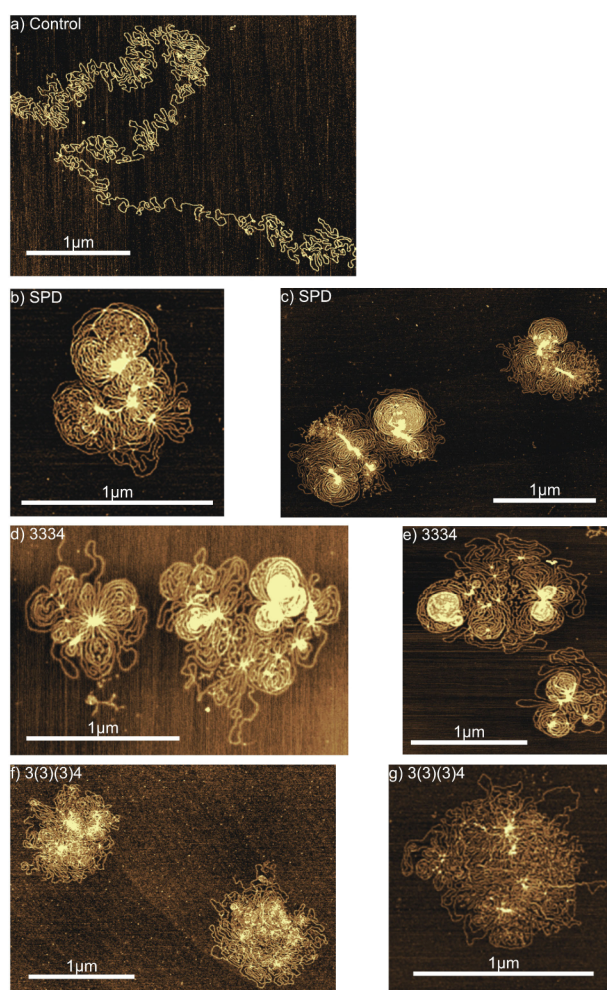
**Scheme 1.** Chemical formulas of linear- and branched-chain polyamines examined in the present study.

while DNA fragments shorter than the persistence length do not form such ordered structures.<sup>[34]</sup>

## 2. Results and Discussion

### 2.1. AFM Observation of the Higher-Order Structure of DNA at Room Temperature

Figure 1 shows typical AFM images of T4 GT7 DNA in the presence of linear- or branched-chain polyamines on a mica surface at 24 °C. The mica surface was not pretreated with any polycations such as magnesium or SPD before the application of a droplet of sample. In this experiment, we adopted 200 μM SPD, 5 μM 3334 and 3 μM 3(3)(3)4 as the polyamine concentrations based on the results obtained through a single molecule observation by fluorescence microscopy (see Supporting Information). The average long-axis length of DNA was ca. 0.4 μm for all polyamines (Figure S1 in the Supporting Information). We have confirmed that the degree of shrinking on the higher-order structure of DNA molecules are essentially the same under the conditions of 200 μM SPD, 5 μM 3334 and 3 μM 3(3)(3)4. Figure 1a shows an elongated conformation of DNA in the presence of a low concentration of SPD (10 μM) as a control observation. In the presence of a linear-chain polyamine, SPD (200 μM) or 3334 (5 μM), DNA molecules with multiple loops tend to crossover at the same point and produce flower-like structures (Figures 1b–1e). Similar DNA conformations induced by SPD were reported by Fang and Hoh.<sup>[37]</sup> On the other hand, in the presence of 3 μM 3(3)(3)4, a mesh-like structure with a number of crossings or bridges appears. In other words, DNA segments are aligned parallel to each other in the presence of a linear-chain polyamine but are randomly-



**Figure 1.** AFM images of T4 DNA in the presence of polyamines at 24 °C: (a) control image at a low concentration, 10 μM SPD; (b) and (c) 200 μM SPD; (d) and (e) 5 μM 3334; (f) and (g) 3 μM 3(3)(3)4. Scale bar: 1 μm. The DNA concentration is 0.5 μM in nucleotide units.

oriented in the presence of a branched-chain polyamine. This difference between linear- and branched-chain polyamines is in good agreement with our previous result.<sup>[31]</sup> To compare the frequency of crossings in the presence of linear- and branched-chain polyamines, we calculated the number of crossings per 1 μm,  $\xi$ , from AFM images based on the method of analysis depicted in Table 1. We counted the number of crossings  $N$  for the total length  $L$  of DNA for the region where the density of DNA segments is not so high and is similar among AFM images with different polyamines. The number of crossings per 1 μm is thus obtained as  $\xi = N/L$ . From the data shown in Table 1, the degree of crossing for 3(3)(3)4 is more than twice those for SPD and 3334.

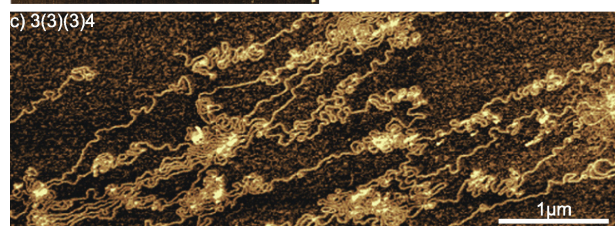
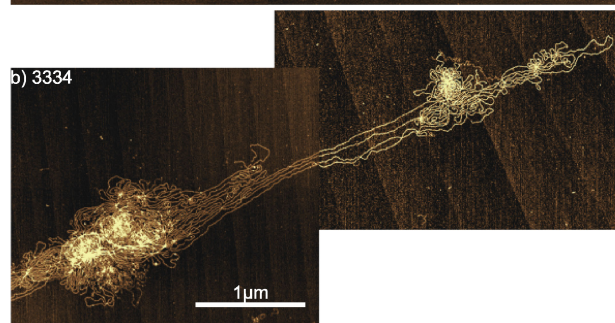
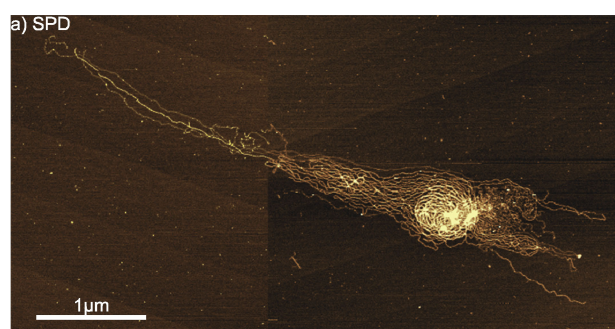
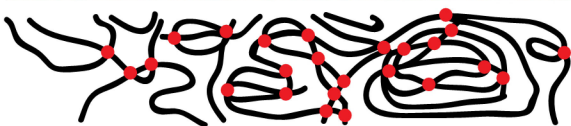
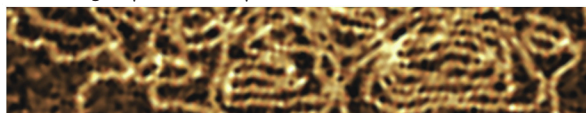
### 2.2. Temperature-Dependent Changes in the Higher-Order Structure of DNA Observed by AFM

Figure 2 shows AFM images of DNA in the presence of polyamines on a mica surface at 50 °C; DNA molecules tend to

**Table 1.** Degree of entanglement  $\xi$ , for a DNA chain evaluated from AFM images, where  $\xi$  represents the number of crossings per 1  $\mu\text{m}$  of DNA chain as depicted schematically in the lower part.

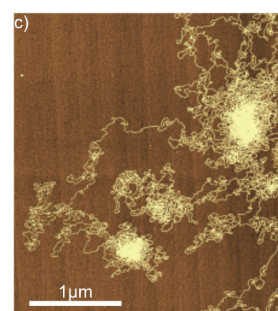
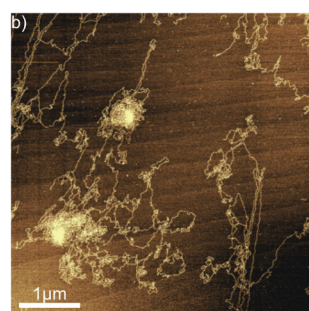
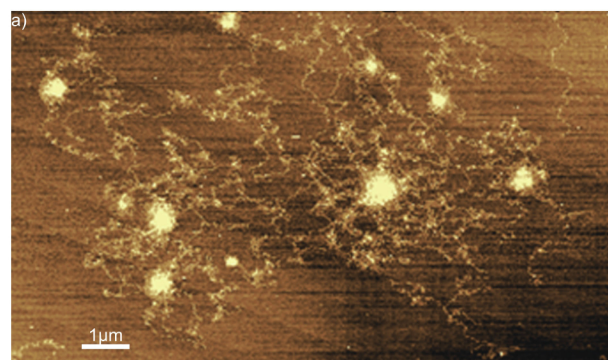
Polyamine	$\xi[\mu\text{m}^{-1}]$	$L/S[\mu\text{m}^{-1}]$
SPD	$5.8 \pm 0.2$	$62.7 \pm 2.2$
3334	$5.7 \pm 0.4$	$59.4 \pm 5.8$
3(3)(3)4	$11.5 \pm 0.3$	$63.7 \pm 4.0$

$\xi$ : number of crossings per 1  $\mu\text{m}$   $N$ : number of crossings,  
 $L$ : total length [ $\mu\text{m}$ ],  $S$ : area [ $\mu\text{m}^2$ ]

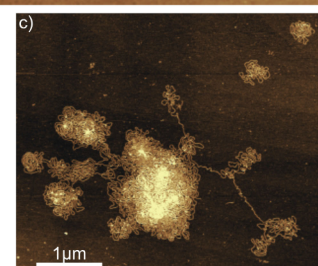
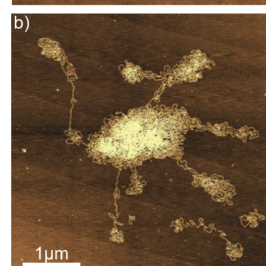
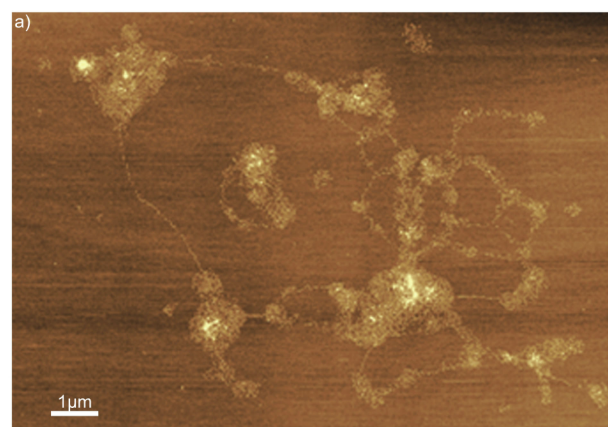


**Figure 2.** AFM images of T4 DNA in the presence of polyamines at 50 °C: (a) 200  $\mu\text{M}$  SPD; (b) 5  $\mu\text{M}$  3334; (c) 3  $\mu\text{M}$  3(3)(3)4.

unwind with increasing temperature. As the temperature rose to 80 °C, there was a marked difference between DNA structures induced by linear- and branched-chain polyamines (Figures 3–5). Especially, the unwounded parts in 3(3)(3)4-induced structure are noted (Figures 5b and 5c). The high-magnification AFM image in Figure 5d clearly indicates that multiple nano-loop structures with diameters of 10–50 nm are

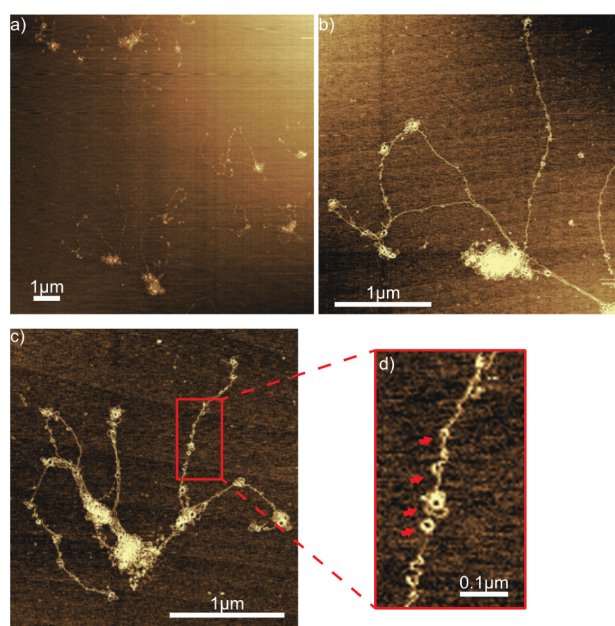


**Figure 3.** AFM images of T4 DNA in the presence of 200  $\mu\text{M}$  SPD at 80 °C: (a) wide scan image; (b) and (c) enlarged images.



**Figure 4.** AFM images of T4 DNA in the presence of 5  $\mu\text{M}$  3334 at 80 °C: (a) wide scan image; (b) and (c) enlarged images.

formed along the DNA strand in the presence of 3(3)(3)4. Such structural features at 80 °C generated by 3(3)(3)4 may be related to its ability to enhance the thermal stability of DNA. The observed nano-looping conformation is similar in size to the inner diameter of a toroid in the tightly folded state of DNA,



**Figure 5.** AFM images of T4 DNA in the presence of 3  $\mu\text{M}$  3(3)(3)4 at 80  $^{\circ}\text{C}$ : (a) wide scan image; (b) and (c) enlarged images; (d) magnified image of the red square in (c), where arrows indicate the appearance of nano-loops.

where the outer diameter is 50–80 nm.<sup>[32–36]</sup> Here, we will roughly estimate the energy cost for the formation of such a nano-loop structure. It has been well established that the persistence length,  $L_p$ , of double-stranded DNA is around 50 nm.<sup>[38]</sup> Under the Hooke's law, the bending elastic constant,  $B$ , has the following relationship with  $L_p$ .<sup>[39]</sup>

$$B = k_B T L_p \quad (1)$$

where  $k_B$  is Boltzmann constant and  $T$  is absolute temperature. Thus, the energy cost,  $\Delta E$ , to form a loop with diameter  $D$  is given as in Eq. (2).

$$\Delta E = 2\pi B/D = 2\pi k_B T L_p/D \quad (2)$$

From this relationship, the energy cost to form a loop of  $D = 10$  nm (with ca. 100 bp DNA or with ca. 200 phosphate groups) is estimated to be  $10\pi k_B T$  ( $\approx 80$  kJ/mol). It is considered that stabilization energy caused by the binding of a single polyamine to phosphate groups is in the order of 10 kJ/mol or more. Thus, an energy cost of  $10\pi k_B T$  to form a 10 nm loop is regarded to be within the range of stabilization energy that can be afforded through the binding of plural number of branched-chain polyamine to the nano-loop.

### 2.3. Effect of Temperature on the Secondary Structure of DNA as Evaluated by CD Measurements

Figure 6 shows the effect of temperature on CD spectra of calf thymus DNA in the presence of linear- and branched-chain polyamines. The CD spectra with different polyamine concentrations were given in the Supporting Information (Figure S2).

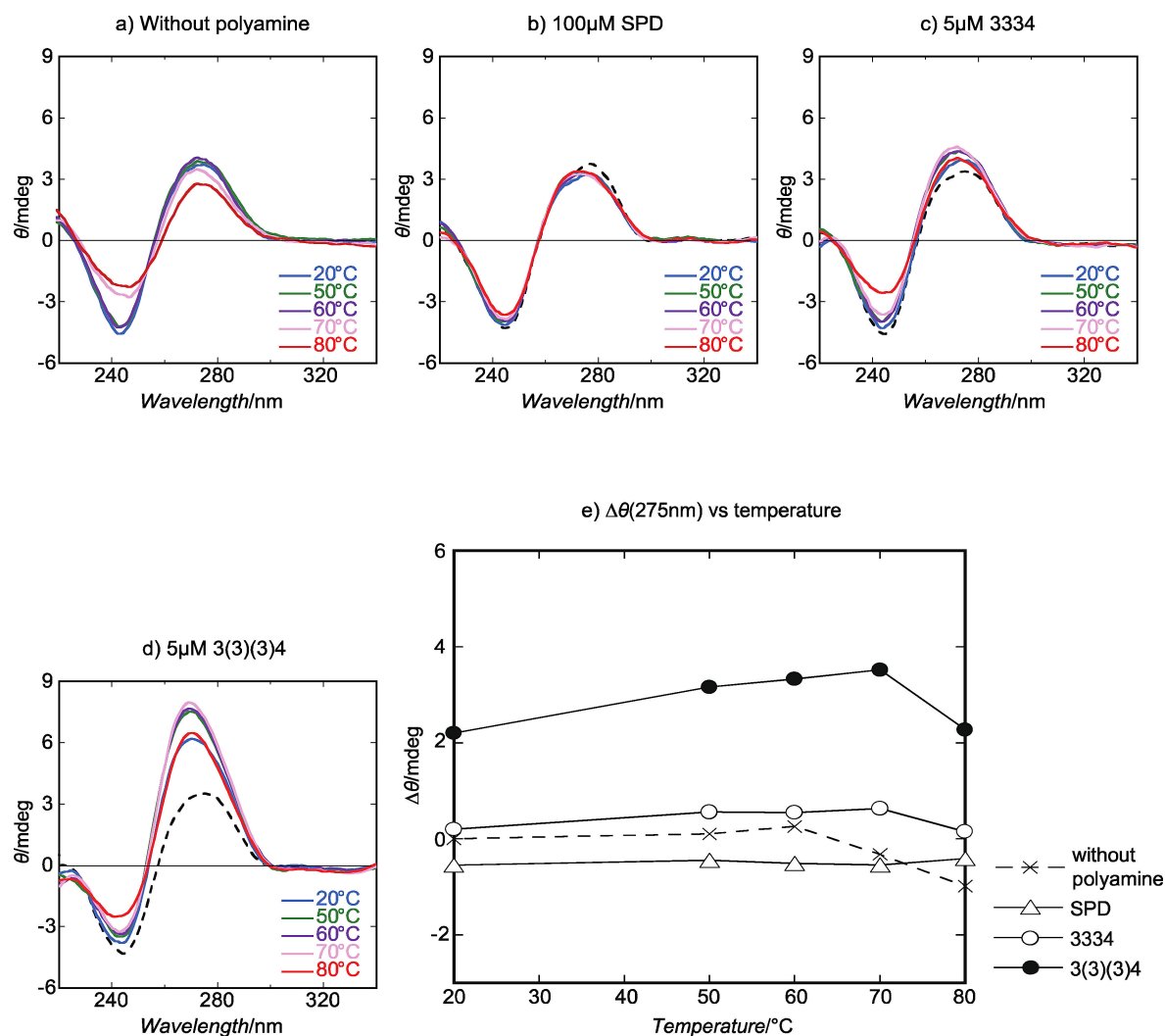
Without polyamine, the CD signals at 240 nm and 275 nm decreased with an increase in temperature to 80  $^{\circ}\text{C}$ , indicating the occurrence of a helix-coil transition (Figure 6a). In the presence of the linear-chain polyamine, SPD, the profiles of CD spectra remained essentially the same from 20  $^{\circ}\text{C}$  to 80  $^{\circ}\text{C}$ , indicating that the secondary structure retains the B-form even at 80  $^{\circ}\text{C}$ . (Figure 6b). This is due to the protective effect of polyamines against the thermal helix-coil transition. The linear-chain polyamine, 3334, was also associated with minimal changes in the CD spectra (Figure 6c). On the other hand, the branched-chain polyamine, 3(3)(3)4, induced a transition from B-form to A-like form DNA characterized by a higher intensity of the positive band at 275 nm over a wide range of temperatures (Figure 6d).<sup>[40]</sup> To better understand the changes in the CD spectrum induced by an increase in temperature, we calculated the difference in ellipticities at 275 nm,  $\Delta\theta$ , between ellipticities obtained in the absence of polyamine,  $\theta_0$  and those obtained in the presence of polyamines,  $\theta_r$ , as defined by the following equation:

$$\Delta\theta = (\theta_r - \theta_0) \quad (3)$$

The change in  $\Delta\theta$  at 275 nm depending on the temperature is depicted in Figure 6e, which shows a marked change in CD with the addition of 3(3)(3)4 even at 20  $^{\circ}\text{C}$ . The value  $\Delta\theta$  tends to increase slightly with an increase of temperature in the presence of 3(3)(3)4. In contrast, the minimal effect of the linear polyamines, SPD and 3334, indicates that the secondary structure retains the B-form even under high temperatures. It was reported that a virus that infects a hyperthermophile, which lives at 80  $^{\circ}\text{C}$  and pH 3, encapsidates A-form DNA.<sup>[41,42]</sup> Whelan et al. suggested that the A-form is prevalent in cells under stress based on observations of live bacteria by FTIR spectroscopy.<sup>[43]</sup> At present, the mechanisms underlying the change from the B-form to A-like form of DNA induced by the branched-chain polyamine 3(3)(3)4 remain unclear, but the preference for the A-like form over the B-form at higher temperatures suggests that the A-like form is associated with thermal resistance.

## 3. Conclusions

Past studies showed the effect of various polyamines on thermal stability of DNA oligonucleotides.<sup>[44,45]</sup> Here we used a large genome-size DNA (166 kbp) and showed that the branched-chain polyamine induces a unique temperature-dependent structural change. To the best of our knowledge, this is the first report to investigate the effect of polyamines on the higher-order structure of large DNA with increasing temperature up to 80  $^{\circ}\text{C}$ . In addition, the branched-chain polyamine induces the secondary structural change from B-form to A-like form that is more marked at higher temperatures. These effects are specific to the branched-chain polyamine and are clearly different from the effects of linear-chain polyamines. These findings in the present study will provide additional insights not only on thermo-adaptation but also on understanding the



**Figure 6.** CD spectra of calf thymus (CT) DNA at different temperatures in the presence of polyamines. The black broken line in (b), (c) and (d) indicates the CD spectrum at 20 °C in the absence of polyamine. The concentration of CT DNA is 30  $\mu\text{M}$  in nucleotide units.

mechanism how hyperthermophiles maintain their genetic activity under high temperature environments.

## Experimental Section

### Materials

Spermidine SPD was purchased from Nacalai Tesque (Kyoto, Japan). Homocaldopentamine 3334 and *N*<sup>4</sup>-bis(aminopropyl)spermidine 3(3)(3)4 were synthesized according to a previous report.<sup>[31]</sup> Calf Thymus DNA (CT DNA: 8–15 kbp) was purchased from Wako Pure Chemical Industries (Osaka, Japan). T4 GT7 phage DNA was purchased from Nippon Gene (Toyama, Japan). Other chemicals were analytical grade and obtained from Nacalai Tesque.

### AFM Observations

AFM measurements were performed with an SPM-9700 (Shimadzu, Kyoto, Japan). For the preparation of sample specimens, 0.5  $\mu\text{M}$  T4 DNA was dissolved in 1 mM Tris-HCl buffer solution at pH 7.5. Polyamines were then added to the solution. The resulting DNA

solution was incubated at the target temperature for 3 min. Freshly cleaved mica was also incubated at the same target temperature. The DNA solution was then transferred onto the mica surface and was stood for 10 min at room temperature (24 °C). Subsequently, the sample was rinsed with ultra-pure water, dried with nitrogen gas and imaged by AFM. All measurements were performed in air using the tapping mode. The cantilever, OMCL-AC200TS-C2 (Olympus, Tokyo, Japan), was 200  $\mu\text{m}$  long with a spring constant of 9–20 N/m. The scanning rate was 0.4 Hz and images were captured using the height mode in a 512  $\times$  512 pixel format. The obtained images were plane-fitted and flattened by the computer program supplied with the imaging module.

### CD Measurements

CD spectra of CT DNA upon heating were measured in the presence of each polyamine in 1 mM Tris-HCl buffer (pH 7.5) on a J-720W spectropolarimeter (JASCO, Tokyo, Japan). The DNA concentration was 30  $\mu\text{M}$  in nucleotide units for all of the CD measurements. The cell path length was 1 cm. Data were collected every 1 nm between 220 and 340 nm at a scan rate of 100 nm/min, and were accumulated 3 times.

## Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 15H02121 and 25103012.

## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** higher-order structure of DNA · branched polyamine · archaea · high temperature · nano-loop

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Manuscript received: April 28, 2018  
Accepted Article published: June 21, 2018  
Version of record online: July 10, 2018