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Site-Directed Spin Labeling of DNA Reveals Mismatch-Induced Nanometer Distance Changes between Flanking Nucleotides

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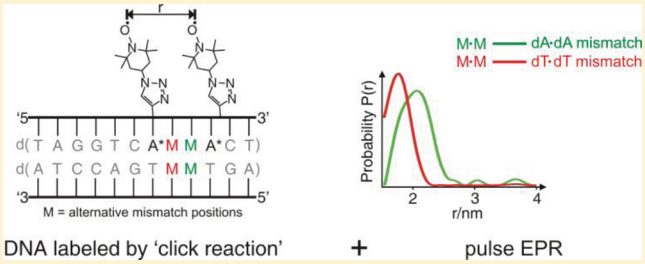
Supporting Information

ABSTRACT: Multiple forms of DNA damages such as base modifications, double-strand breaks, and mispairings are related to inheritable diseases, cancer, and aging. Here, the structural changes of duplex DNA upon incorporation of mismatched base pairs are examined by EPR spectroscopy. Two ethynyl-7-deaza-2′-deoxyadenosine residues separated by two nucleotides were incorporated in DNA and functionalized with 4-azido-2,2,6,6-tetramethyl-piperidine-1-oxyl (4-azido TEMPO) by the click reaction. Mismatches such as dT·dT or dA·dA mispairs were positioned between these two spin labels in DNA duplexes. Pulse EPR experiments reveal that the mismatch-induced local conformational changes are transmitted to the flanking nucleotides and that the impact of this mismatch depends on the nearest neighbor environment.



INTRODUCTION

2′-Deoxyribonucleic acid (DNA) is the carrier of genetic information in living organisms. DNA mutations such as nucleobase changes, double-strand breaks, and mispairings are associated with inheritable diseases, cancer, and aging.[1−3] In vivo, DNA mismatches are usually caused by the misincorporation of nucleotides during replication[4] or mutagenesis caused by chemical compounds[5,6] or ionizing radiation.[7] This can lead to conformational changes of DNA domains with biological impact in respect of mutagenicity. Consequently, such alterations, if not repaired, have severe effects on the cellular machinery. Efficient initiation of the cellular mismatch repair system requires the discrimination between intact and damaged DNA sites by the repair machinery. A key component in the cellular repair system is the protein MutS, which has the capability to scan DNA for mismatches thereby testing the shape and flexibility of DNA.[8] The repair efficiency seems to correlate with the induced conformational changes rather than with the thermodynamic stability of the mismatches.[9,10]

Conformational changes can be monitored by intramolecular distance measurements, which provide new insights into structure−function relationships.[11−13] Nanometer distance measurements related to mutagenic events involving DNA structural changes have been determined by high-resolution NMR spectroscopy,[14] fluorescence resonance energy transfer (FRET),[15] and X-ray crystallography.[16,17] Recently, the high sensitivity and accuracy of pulse electron double resonance (PELDOR, or double electron−electron resonance, DEER) on spin labeled oligonucleotides have been demonstrated under conditions close to the physiological state.[18−21] Several techniques have been developed for the spin labeling of nucleic acids. The highly exergonic character of the copper(I)-catalyzed Huisgen−Sharpless−Meldal alkyne−azide cycloaddition (CuAAC), the so-called click reaction, was used for spin labeling of DNA on solid-phase by Sigurdsson and coworkers[22] and in solution by the Seela and Steinhoff groups.[23] A 7-ethynyl derivative of 7-deaza-2′-deoxyadenosine (**1**) was incorporated in an oligonucleotide, followed by the click functionalization with 4-azido-2,2,6,6-tetramethyl-piperidine-1-oxyl (**2**; 4-azido TEMPO) leading to derivative **3** (Figure 1).[23] This reaction occurred with high efficiency without perturbing the DNA duplex structure. PELDOR experiments revealed an exceptional narrow distance distribution, which enables identification of even small mismatch-induced structural changes of oligonucleotides. However, rigid spin labels may lead to a specific mutual orientation of both reporter groups.[24−26] Therefore, the determination of interspin distance distributions under the assumption of an isotropic distribution of spin label orientations, as applied in common data analysis programs, may be inaccurate. Consequently, the PELDOR spectra and the derived distance distributions might be modulated by this effect. A study by Schiemann et al. using compound Ç as spin label, first introduced by Barhate et al.,[27] revealed that an accurate determination of distances and

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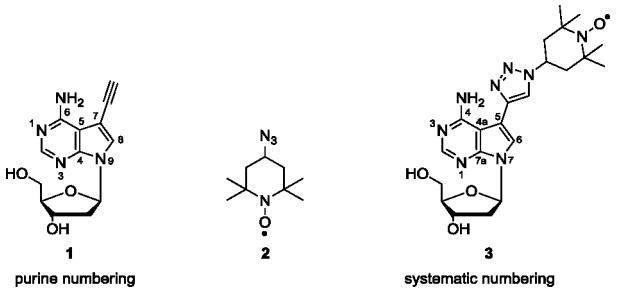
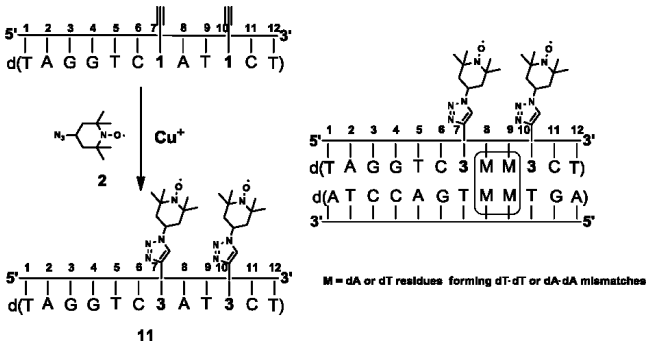


Figure 1. Structures of 7-ethynyl-7-deaza-2′-deoxyadenosine and 4-azido TEMPO conjugates.

relative orientations of spin centers in nucleic acids can be achieved by orientation selective PELDOR experiments at X-band frequencies.[24]

Here, we provide insight into conformational changes of DNA caused by mismatch formation. To this end, orientation selective PELDOR was used to examine the changes in the structure and dynamics of fully matching as well as mispaired spin labeled DNA duplexes. Mismatches were incorporated into oligonucleotides containing nucleoside derivative **1** as reactive group and the 4-azido TEMPO spin label **2** was introduced by the click reaction. Four mismatched constructs with dT·dT and dA·dA pairs located between two spin labeled positions (Table 1 and Scheme 1) were synthesized and analyzed. The results

Scheme 1. Spin Labeling of DNA by Click Reaction and Formation of Duplexes Containing One dT·dT or dA·dA Mismatch in Positions 8 or 9



reveal that changes of the interspin distances appear depending on the type of the incorporated mismatches and nearest neighbor environment.

MATERIALS AND METHODS

Synthesis and Characterization of Oligonucleotides for Spin Labeling. The protocols of synthesis and characterization of oligonucleotides have been described.[23,28] The functionalization of oligonucleotides with 4-azido-2,2,6,6-tetramethyl-piperidine-1-oxyl (**2**) using the Huisgen–Sharpless–Meldal alkyne–azide cycloaddition (CuAAC) was reported previously.[23] The oligonucleotides were purified by reversed-phase HPLC and characterized by MALDI-TOF mass spectrometry and enzymatic hydrolysis. The melting curves were measured with a Cary-100 Bio UV–vis spectrophotometer (Varian, Australia) equipped with a Cary thermoelectrical controller. The temperature was measured continuously in the reference cell with a Pt-100 resistor with a heating rate of 1 °C min^{-1}. The thermodynamic data of duplex formation were calculated by the Meltwin 3.0 program.

Pulse EPR Experiments. For all pulse EPR experiments, 30–40 μL of the sample solution in 0.1 M NaCl and 10 mM $MgCl_2$ with a DNA concentration of 60 μM was loaded into EPR quartz capillaries with 3 mm outer diameter. 10% glycerol was added as cryoprotectant. The sample was frozen in liquid nitrogen before insertion into the resonator. Pulse EPR measurements were performed at 50 K and X-band frequencies (~9.4 GHz) using a Bruker Elexsys 580 spectrometer equipped with a 3 mm split ring resonator (ER 4118X-MS3, Bruker). A continuous flow cryostat (ESR900, Oxford Instruments) in combination with a temperature controller (ITC 503S, Oxford Instruments) was used for temperature stabilization. The four-pulse PELDOR sequence[29] was applied. For orientation selective experiments, the observer frequency υ_{obs} was varied to achieve frequency offsets of 40, 65, and 80 MHz between observer and pump pulses. All the other parameters were kept unchanged with respect to the previously specified values.[23]

The determination of interspin distances in the range of 1.5–8 nm is based on the dipolar coupling frequency of dipolar coupled spins. The fitting of the background corrected dipolar evolution function using Tikhonov regularization as implemented in DEERAnalysis2008[30] requires the excitation of all spin label orientations. Therefore, the three orientation selective PELDOR traces were normalized with respect to the number of scans, summed up (orientation averaging) utilizing the Xepr software (Bruker), and analyzed by means of DEERAnalysis2008[30] (cf. Figure 2). The error of the distance values were evaluated by the validation tool included in

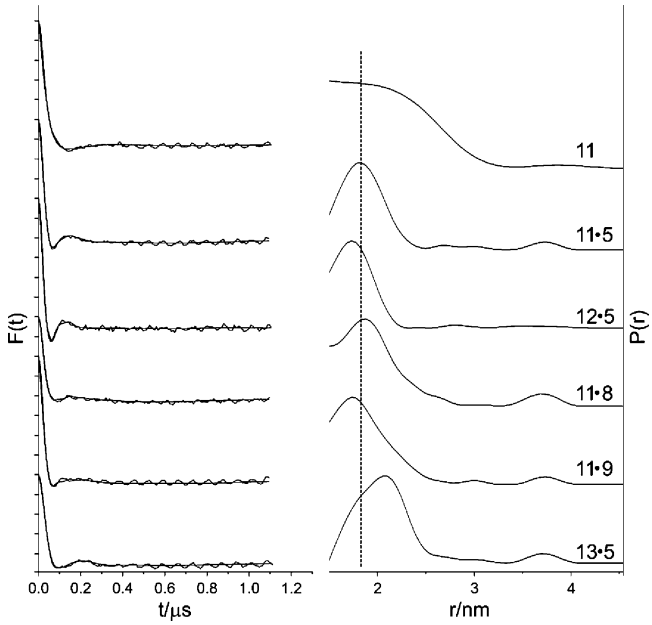


Figure 2. Averaged PELDOR traces (9.4 GHz) measured at 50 K (see Materials and Methods for details). Left: background corrected dipolar evolution data, $F(t)$; tick marks are separated by 0.05. Right: distance distributions, $P(r)$, obtained by Tikhonov regularization (DeerAnalysis2008).[30] Weak contributions from proton modulations are visible with a frequency of about 14.6 MHz. Data analysis and distance distributions are not significantly affected, as the proton modulation frequency would correspond to a distance of about 1.5 nm. To guide the eye, a dashed line is depicted at the position of the r_{peak} value of the perfectly matched DNA duplex **11·5** (r_{peak} = 1.83 nm).

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DEERAnalysis. Extra noise was added with a noise factor of 1.5, and the number of trials amounted to 50. For each distance distribution, the distance value, where the probability showed a maximum (r_{peak}), was determined, and the standard deviation was calculated for the respective double stranded DNA duplex. No error value was calculated for the single stranded DNA oligonucleotide due to its broad distance distribution width.

RESULTS AND DISCUSSION

Introduction of Nitroxide Spin Labels and Mismatches into Oligonucleotides. A series of oligonucleotides was synthesized by solid-phase phosphoramidite synthesis, deblocked under mild deprotection conditions, and purified by reversed-phase HPLC. Ethynylated oligonucleotide strands **6**, **7**, and **10** were functionalized with 4-azido-2,2,6,6-tetramethylpiperidine-1-oxyl (**2**) as reported previously using copper(I) catalyzed Huisgen−Sharpless−Meldal alkyne−azide cycloaddition (CuAAC).[23] The formation of click products was confirmed by MALDI-TOF mass spectrometry and enzymatic hydrolysis (see Supporting Information, Table S1 and Figure S1). The spin labeled single strands were hybridized with unmodified second strands in solution of low salt concentration (0.1 M NaCl, 10 mM $MgCl_2$, and 10% glycerol) yielding a set of duplexes with canonical base pairs (**11·5**) or containing dT·dT or dA·dA mismatches (**12·5**, **11·8**, **11·9**, and **13·5**). So, two bidentate dA·dT base pairs are located in the center of the duplex between two spin labeled positions. Tridentate dC·dG base pairs surround the spin labels at both sites (positions 6 and 11), thereby stabilizing the local structure. These positions were chosen to separate the impact of changes of the microenvironment of the spin label from those introduced by mispairings. Effects induced by mispairing and hybridization are further distinguished by investigation of double labeled single stranded (**11**) and double stranded DNA (**11·5**).

The formation of mismatches does not only cause changes of the thermodynamics and kinetics of a particular canonical base pair but also affects nearest neighbors. Consequently, the nearest neighbor model for Watson−Crick base pairs was extended to interactions between mismatches and neighboring base pairs.[31,32] It was shown that with the exception of terminal positions the thermodynamics of a mismatch is independent of its position within a particular duplex but depends on the local environment of the mismatch. A thermodynamic analysis was performed for the perfectly matching and mismatched ethynylated duplexes (Table 1). For comparison, the T_m values of the duplexes containing one mismatched pair in between the spin labels are presented (Table 2). To be consistent with the conditions utilized in low temperature EPR experiments, 10% glycerol was added to the solution throughout all T_m measurements. As shown in Table 1, the replacement of two dA residues by 7-ethynyl-7-deaza-2′-deoxyadenosine (**1**) has a positive effect on duplex stability: the T_m value rises by 4 °C for **6·5** compared to **4·5**. However, the introduction of a dA·dA or dT·dT mismatch in between the ethynylated residues results into a pronounced duplex destabilization (ΔT_m = −(11−14) °C), in agreement with the former observation that the presence of dA·dA or dT·dT mismatches in the center of a duplex reduced the T_m value significantly.[10,33,34]

The introduction of the two spin labels into the perfectly matching DNA duplex (**11·5**) resulted in a T_m value of 41 °C (Table 2), which is only slightly decreased compared to the unmodified DNA duplex **4·5** (T_m = 43 °C). This is evidence for a nearly undisturbed helix structure due to the uncritical

Table 1. T_m Values of Oligonucleotide Duplexes Containing Ethynyl-Substituted Nucleosides[a]

	duplex	T_m (°C)	ΔT_m (°C)[b]
4·5	5′-d(TAG GTC AAT ACT)-3′ (**4**)	43	
	3′-d(ATC CAG TTA TGA)-5′ (**5**)		
6·5	5′-d(TAG GTC **1**AT **1**CT)-3′ (**6**)	47	
	3′-d(ATC CAG TTA TGA)-5′ (**5**)		
7·5	5′-d(TAG GTC **1**TT **1**CT)-3′ (**7**)	36	−11
	3′-d(ATC CAG TTA TGA)-5′ (**5**)		
6·8	5′-d(TAG GTC **1**AT **1**CT)-3′ (**6**)	35	−12
	3′-d(ATC CAG TTT TGA)-5′ (**8**)		
6·9	5′-d(TAG GTC **1**AT **1**CT)-3′ (**6**)	33	−14
	3′-d(ATC CAG TAA TGA)-5′ (**9**)		
10·5	5′-d(TAG GTC **1**AA **1**CT)-3′ (**10**)	33	−14
	3′-d(ATC CAG TTA TGA)-5′ (**5**)		

[a]Measured at 260 nm in an aqueous 0.1 M NaCl solution containing 10 mM $MgCl_2$ and 10% glycerol with 5 μM single-strand concentration. [b]ΔT_m was calculated as T_m(mispaired DNA duplex) − T_m(**6·5**).

positions of the spin labels at C-7 of the 7-deazapurine moiety. The substitution of a dA·dT base pair by a dT·dT mispair (duplex **12·5**) results into a pronounced duplex destabilization with ΔT_m = −12 °C compared to the perfectly matching DNA duplex **11·5**. A similar result was obtained for duplex **11·8** containing the dT·dT mismatch close to the second spin label toward the 5′-end of the duplex, ΔT_m = −13 °C. Even more pronounced destabilizations were found in the case of the dA·dA mismatches. The T_m values are decreased by 17−19 °C (T_m = 22 °C for **11·9**; T_m = 24 °C for **13·5**).

Interspin Distance Changes Reveal Mismatch-Induced Conformational Changes. Misincorporations of nucleotides, if not repaired, have severe effects on the cellular machinery. Such alterations can lead to changes of the DNA conformation, which in turn may result in twists, bending, shortening, or elongation of the DNA double helix. PELDOR has been shown to provide valuable information on the impact of mispairings on the distances between spin labeled nucleotides. Sicoli et al.[12] reported on interspin distance changes in duplex DNA induced by lesions. In those experiments, the two spin labels were located in the minor groove and separated by six nucleotides containing the lesion. Kuznetsov et al.[35] presented experiments on DNA containing non-nucleotide inserts with spin labels bound at the 3′-end and 5′-end phosphate groups separated by 12 nucleotides and suggested a 20° bending of the double helix to occur. The large separation of the spin labels in those experiments favors the detection of lesion-induced global conformational alterations, which are accompanied by internucleotide distance changes along the double helix axis, i.e., shrinking, elongation, or bending of the double strand. However, a large separation of the two spin labels from the lesion sites renders their distance value less sensitive to helix twists or only local conformational changes. In the present study, the two spin labels of the 12-mer duplex DNA are located in the major groove and separated by only two nucleotides. Hence, the present experiments are expected to be sensitive to local changes due to base pair overlaps or base pair twists, which could lead to alterations of interspin distances perpendicular to the helix axis.

PELDOR data depend on the mutual orientation of the spin label molecules. Orientation correlation can be neglected if the spin label side chains are sufficiently flexible. However, if the

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Table 2. T_m Values of Oligonucleotide Duplexes Containing Spin Labeled Conjugates[a] and Parameters for the Interspin Distance Distributions, r_{peak} and w, Determined by PELDOR Experiments

	duplex	T_m (°C)	ΔT_m (°C)[b]	r_{peak} (nm)[c]	w (nm)
4·5	5′-d(TAG GTC AAT ACT)-3′ (**4**)	43			
	3′-d(ATC CAG TTA TGA)-5′ (**5**)				
11	5′-d(TAG GTC **3**AT**3** CT) (**11**)			2.0	>1
11·5	5′-d(TAG GTC **3**AT**3** CT) (**11**)	41		1.83 ± 0.02	0.5
	3′-d(ATC CAG TTAT GA) (**5**)				
12·5	5′-d(TAG GTC **3**TT**3** CT) (**12**)	29	−12	1.73 ± 0.02	0.5
	3′-d(ATC CAG TTAT GA) (**5**)				
11·8	5′-d(TAG GTC **3**AT**3** CT) (**11**)	28	−13	1.87 ± 0.03	0.6
	3′-d(ATC CAG TTTT GA) (**8**)				
11·9	5′-d(TAG GTC **3**AT**3** CT) (**11**)	22	−19	1.73 ± 0.03	0.7
	3′-d(ATC CAG TAAT GA) (**9**)				
13·5	5′-d(TAG GTC **3**AA**3** CT) (**13**)	24	−17	2.08 ± 0.03	0.7
	3′-d(ATC CAG TTAT GA) (**5**)				

[a]Measured at 260 nm in an aqueous 0.1 M NaCl solution containing 10 mM $MgCl_2$ and 10% glycerol with 5 μM single-strand concentration. [b]ΔT_m was calculated as T_m(mispaired DNA duplex) − T_m(**11·5**). [c]Error values were obtained utilizing the validation tool included in DEERAnalysis2008[30] (see Materials and Methods for details).

spatial freedom of the spin label side chains is restricted, only a few rotameric states may be populated and PELDOR data recorded at different frequency offsets can provide additional information about the relative spin label orientations even at X-band (~9 GHz) frequencies (see, e.g., ref 36 and references therein). However, distance distributions calculated from these PELDOR traces under the assumption of an isotropic distribution of spin label orientations may lead to misinterpretation. In this case, averaging of the orientation selection effect has been shown to yield reasonable results.[37,38] Marko et al.,[38] e.g, showed that distance distributions determined by Tikhonov regularization using the average over PELDOR time traces with different frequency offsets in the range from 40 to 80 MHz were in excellent agreement with results from molecular dynamics simulations. Hence, PELDOR traces of the spin labeled DNA constructs were recorded at frequency offsets of $\Delta\nu$ = 40, 65, and 80 MHz. The individual PELDOR traces and their averages were analyzed using DEERAnalysis2008.[30] The distance distributions were characterized by the distance value, r_{peak}, where the probability shows a maximum, and by their widths, w, as a measure of the conformational heterogeneity. The distance values, r_{peak}, determined from the individual orientation selective PELDOR traces of each sample agree within experimental error (<0.03 nm) with that calculated from the corresponding averaged PELDOR trace. This is evidence for the absence of any gross angular or orientation−distance correlation between the two spin labels in the present samples.

The results (Figure 2, Table 2) discussed in the following are based on the average of the orientation selective PELDOR traces. The interspin distance distribution for the single stranded DNA **11** is centered at 2 nm with a distribution width exceeding 1 nm. Hybridization or introduction of mismatches significantly changes the interspin distance distributions. The double stranded, perfectly matching DNA duplex **11·5** exhibits an interspin distance of 1.83 ± 0.02 nm and a significantly smaller distribution width of 0.5 nm. Compared to single stranded DNA, the more structured conformation due to duplex formation is readily seen in the decrease of the distance distribution widths in agreement with results reported by Kuznetsov et al.[35] Incorporation of the thymine mismatch in the complementary strand at position 8 decreases the mean interspin distance (**12·5**, 1.73 ± 0.02 nm). An almost identical distance distribution is observed for DNA duplex **11·9** carrying a dA·dA mispair at position 8. For the DNA duplex **11·8** with the dT·dT mispair at position 9, the mean interspin distance is enlarged (1.87 ± 0.03 nm). The largest interspin distance (2.08 ± 0.03 nm) is found for DNA duplex **13·5** with the dA·dA mispair at position 9. For all mismatched double labeled and double stranded DNA duplexes, the distribution widths of 0.5−0.7 nm resemble the results for the perfectly matching double labeled DNA duplex **11·5**. In all cases, the simulated form factors $F(t)$ are in excellent agreement with the experimental ones. Further validation of the data was performed by inspection of the experimental and simulated Pake pattern (see Supporting Information, Figure S2).

The observed increase or decrease of the interspin distances is strong evidence that the DNA mismatch site shrinks or expands. The resulting interspin distance changes depend not only on the type of mismatch (dA·dA or dT·dT) but even more pronounced on the mismatch position with respect to the spin labeled nucleotides as illustrated in Figure 3A,B. Hence, our experiments reveal that the mismatch-induced conformational changes are transmitted to the flanking nucleotides and that the impact of this mismatch depends on the nearest neighbor environment. Experimental NMR results[39] revealed that DNA duplexes are not greatly distorted by the introduction of dA·dA or dT·dT mismatches and that their global conformation is that of a canonical B-form double helix in agreement with our finding. Furthermore, it was shown that, for both systems (dA·dA or dT·dT), two conformations with the same donor and acceptor pattern can coexist, which are engaged in rapid exchange, one being obtained from the other by a 180° rotation about the pseudodyadic axis (cf. Figure 3). Our experiments show that these local conformational changes affect in a position dependent manner the distance between nearest neighbors.

■ CONCLUSIONS

This work reports on distance changes occurring in site-directed spin labeled duplex DNA when mismatches replace canonical base pairs. On the basis of ethynylated DNA, 4-azido TEMPO spin labels were introduced at the 7-position of the 7-

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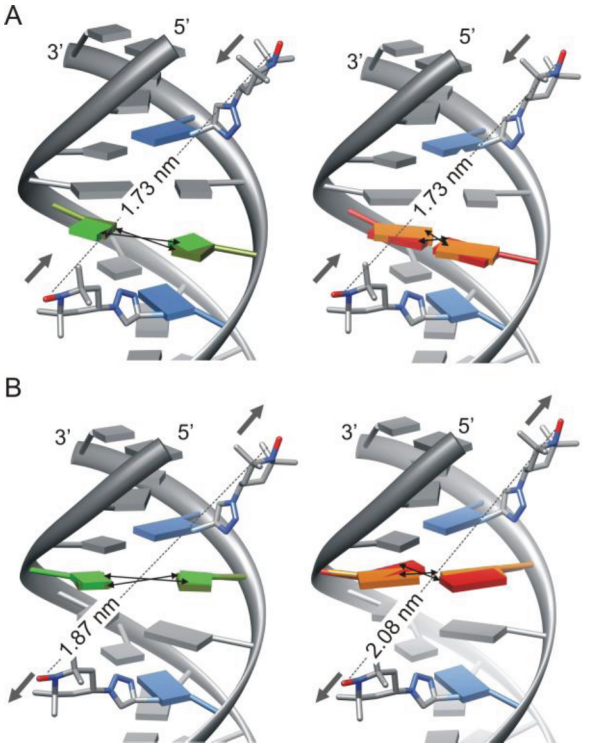

Figure 3. Scheme illustrating the local conformational changes of spin labeled DNA induced by incorporated dT·dT (light and dark green) and dA·dA mispairs (red and orange) at positions 8 (A) and 9 (B). Spin labels were oriented according to results of molecular dynamics simulations of the perfectly matching DNA duplex.[23] The mispairs are engaged in a rapid conformational exchange[39] (black arrows). Experimentally determined interspin distances, r_{peak}, for the mismatched DNA duplexes are depicted in each figure. Compared to the perfectly matching DNA duplex **11·5** (r_{peak} = 1.83 nm), the insertion of mismatches at position 8 (A) decreases the interspin distances, whereas mismatches at position 9 (B) increase the interspin distances (indicated by gray arrows).

deazadenine moiety by the copper assisted azide−alkyne click reaction. The distance between spin labels bound to flanking nucleotides changes when canonical dA·dT base pairs are replaced by dA·dA or dT·dT mispairs. The interspin distances depend not only on the type of mismatch but also on its location with respect to the spin labeled nucleotide. The results disclose local changes induced by the mismatches under participation of the nearest neighbors. These changes are finally transferred to the base pairs carrying the spin labels. This cross-talk cannot be observed when the mismatches are far away from the bases bearing the spin label. Implications of these conformational changes are obvious for DNA repair, as repair enzymes do not simply recognize single mismatches but scan the DNA conformation close to the mismatch site.[8,40]

■ ASSOCIATED CONTENT

Supporting Information

MALDI-TOF mass data of oligonucleotides, enzymatic hydrolysis data, and Pake pattern. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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