DNA Conformations

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Formation of Sequence-Independent Z-DNA Induced by a Ruthenium Complex at Low Salt Concentrations**

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Z-DNA has attracted much attention during the past 30 years.[1] The high binding affinity of some proteins to Z-DNA[2–4] means that these sequences are novel and important regulators of several genes, such as C-MYC, CSF1, and human ADAM-12.[5–7] Liu et al. reported the distribution of Z-DNA sequences and their effects on transcription.[8] It is generally agreed that potential Z-DNA-forming sequences are located near the promoter region of most human genes, and that Z-DNAs upregulate transcription.[9,10] In addition, Z-DNA may affect chromatin recombination and nucleosome positioning.[11]

Various sequences of natural polynucleotides, especially non-alternating pyrimidine–purine (non-APP) or AT-rich sequences, are rarely found in the Z-DNA conformation. Instead, this conformation is favored by alternating GC sequences, which usually contain alternating *syn*-G and *anti*-C nucleosides.[12] AT base pairs that are inserted into Z-DNA sequences destabilize the conformation and induce a DNA cruciform.[13,14] Non-APP segments, especially A_nT_n ($n>2$), cause the DNA to bend into so-called adenine–thymine tracts (AT tracts), which prevent the formation of Z-DNA.[15,16] To date, the number of reported sequences that adopt the Z-DNA conformation is limited, and most of these sequences are GC-rich. In fact, GC-rich Z-DNA usually occurs at extremely high salt concentrations or at a considerable concentration of multivalent cations, such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+}.[17–20] The formation of Z-DNA in solutions with low salt concentrations can be achieved by using methylated or brominated dsDNA; however, these modified nucleotides may not be suitable for biological studies.[21–24] Few small molecules, such as cobalt hexamine, polyamines, and polynuclear platinum complexes, can induce a Z-DNA conformation in GC sequences.[25–27] Qu et al. and Xu et al. reported the chiral selectivity of drug binding with Z-DNA, and the allosteric transition of poly-d(GC), but this phenomenon was observed when 2.25 M NaCl was used.[28,29]

The Z-DNA conformation is a transient state that is rarely formed because of the repulsion within the negatively charged phosphate backbone, the sections of which are much closer to each other in Z-DNA than in B-DNA; Z-DNA is formed occasionally during some biological processes.[30] Naturally occurring poly-d(AT) and TATA box sequences are upstream promoter elements that are required for gene expression.[31] The facile transition of promoter elements, such as AT-rich sequences, from the B to the Z conformation may significantly regulate the biological activity of double-stranded DNA. Nevertheless, few studies on Z-DNA with AT-rich sequences have been reported, most likely because the less stable AT base pairs result in the Z conformation only in low-water-content films.[13,32] For poly-d(AT), the Z-DNA conformation is only observed at a high NaCl concentration and at a Ni^{2+} concentration above 85 mM.[33]

Herein, we report the synthesis of the ruthenium complex[34] $[Ru(dip)_2dppz]^{2+}$ (dip = 4,7-diphenyl-1,10-phenanthroline, dppz = dipyridophenazine), which can efficiently induce the B to Z transition of various DNA sequences, such as non-APP and full-AT sequences (for the synthesis and structure of the complex, see Scheme S1 in the Supporting Information). The similar complex $[Ru(dip)_3]^{2+}$ was studied by Barton et al. in the 1980s; however, this complex acts as a chiral probe for Z-DNA and does not induce Z-DNA formation.[35] Because of the presence of the base-pair-intercalating ligand dppz,[36] $[Ru(dip)_2dppz]^{2+}$ had a high DNA-binding affinity with the ability to induce the Z-DNA conformation in NaCl solution (25 mM) buffered with Tris-HCl (10 mM, pH 7.5). The transition occurred extremely quickly in solution regardless of the nature of the DNA sequences. As the concentration of $[Ru(dip)_2dppz]^{2+}$ increased (Figure 1), the Z form (signal at 283 nm) was generated and the B form (250 nm for full-AT DNA, 248 nm for AT-tract DNA) disappeared gradually; the fact that the titration curves all passed through one point meant that the transition occurred between B and Z conformations.[37] Notably, the full-AT sequences $(AT)_6$, AT tract, $(AT)_{12}$, TATA box, and poly-d(AT), as well as genomic DNA

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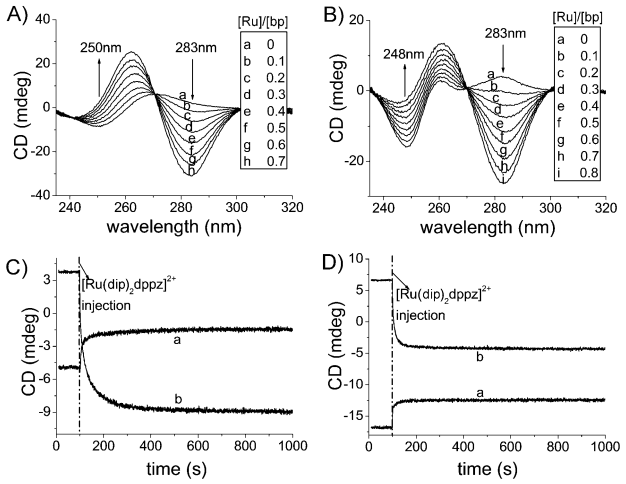

Figure 1. CD spectra of full-AT and AT-tract Z-DNA induced by [Ru(dip)$_2$dppz]$^{2+}$ in a low-salt aqueous solution. CD titrations of A) (AT)$_6$ and B) AT-tract DNA ([bp] = 20 μM) with [Ru(dip)$_2$dppz]$^{2+}$ (A: [Ru] = 2–14 μM, B: [Ru] = 2–16 μM) at 25 °C in a buffered solution (25 mM NaCl, 10 mM Tris-HCl, pH 7.5, Tris = tris(hydroxymethyl)aminomethane). B→Z transition kinetics of C) (AT)$_6$ and D) AT-tract DNA ([bp] = 20 μM) at 4 °C in a buffered solution (25 mM NaCl, 10 mM Tris-HCl, pH 7.5), monitored by CD spectroscopy at a) 285 nm and b) 250 nm, respectively. The DNA solution was stirred and [Ru(dip)$_2$dppz]$^{2+}$ (5 μM) was injected when the signal was stable for 100 s, respectively.

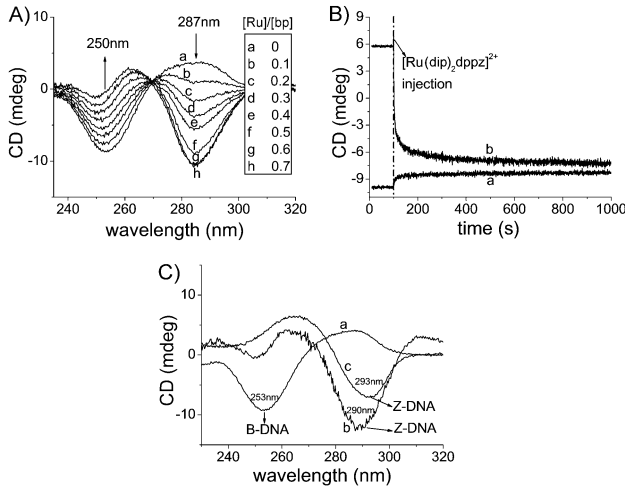

Figure 2. CD spectra of Z-(GC)$_6$ induced by [Ru(dip)$_2$dppz]$^{2+}$ in a low-salt solution. In a high-salt solution (5 M NaCl), unmodified (GC)$_6$ is in the Z conformation. A) CD titration of (GC)$_6$ DNA ([bp] = 20 μM) with [Ru(dip)$_2$dppz]$^{2+}$ ([Ru] = 2–14 μM) at 25 °C in a buffered solution (25 mM NaCl, 10 mM Tris-HCl, pH 7.5). B) B→Z transition kinetics of (GC)$_6$ ([bp] = 20 μM) induced by [Ru(dip)$_2$dppz]$^{2+}$ ([Ru] = 10 μM) at 4 °C in a buffered solution (25 mM NaCl, 10 mM Tris-HCl, pH 7.5), monitored by CD spectroscopy at a) 250 nm and b) 285 nm. C) CD spectra of a) (GC)$_6$ B-DNA, and (GC)$_6$ Z-DNAs induced by b) [Ru(dip)$_2$dppz]$^{2+}$ (25 μM) and c) NaCl (5 M). NaCl-induced Z-DNA was buffered with 10 mM Tris-HCl, pH 7.5, 25 °C. B-DNA and [Ru(dip)$_2$dppz]$^{2+}$-induced Z-DNA were buffered with 25 mM NaCl, 10 mM Tris-HCl, pH 7.5, 25 °C.

and lpBR322 were all efficiently converted into the Z conformation under low-salt conditions (Figures S2 and S3).

To the best of our knowledge, this is the first observation of Z-DNA-containing full-AT and AT-tract sequences under low-salt conditions. Rich and co-workers resolved the crystal structure of Z-DNA that contained AT base pairs,[12] and demonstrated that unmodified A and C bases can adopt a *syn* conformation comparable to that in typical Z-DNA. Therefore, we assigned a significant Cotton effect at a wavelength of 283 nm in the CD spectra to the Z conformation of full-AT DNA. To compare the effect with Z-DNA formed by full-GC sequences, we monitored the CD spectra of unmodified (GC)$_6$ (Figure 2) and poly-d(GC) (Figure S2) under low-salt and high-salt conditions. As expected, a significant negative peak at 290 nm appeared, and the negative peak intensity of B-DNA at 253 nm decreased but remained below 0; this observation indicates the formation of left-handed DNA structures under low-salt conditions. For both the full-AT and full-GC sequences, the transition process reached equilibrium on a time scale of 100 s after the injection of [Ru(dip)$_2$dppz]$^{2+}$. Significant Cotton effects at 283 nm and 290 nm indicated that a Z-DNA conformation was induced. Moreover, Ho demonstrated that the non-B-DNA structure of d(CA/TG)$_n$ did not differ from that of Z-DNA.[38] These results suggest that full-AT sequences and full-GC sequences formed Z-DNA.

To investigate the specific effect of the B to Z transition induced by [Ru(dip)$_2$dppz]$^{2+}$, we synthesized structurally related complexes and carried out comparison experiments. Molecular light switches [Ru(bpy)$_2$dppz]$^{2+}$, [Ru(dip)$_3$]$^{2+}$, and [Ru(dip)$_2$phen]$^{2+}$ reported by Barton and co-workers were synthesized (see Scheme S2 and related references), and the induction of Z-DNA by these four complexes was monitored by CD spectroscopy under similar conditions (Figure 3). Use of complexes [Ru(bpy)$_2$dppz]$^{2+}$, [Ru(dip)$_3$]$^{2+}$, and [Ru(dip)$_2$phen]$^{2+}$ led to few changes in the CD spectra of (AT)$_6$,

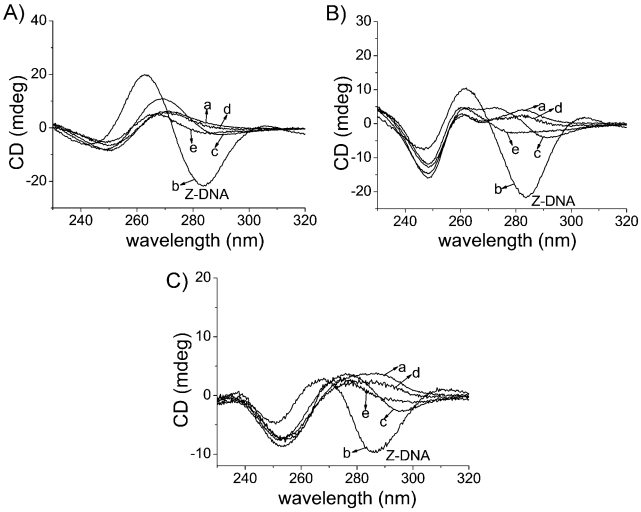

Figure 3. CD spectra of A) (AT)$_6$, B) A$_{24}$T$_{24}$, and C) (GC)$_6$ sequences ([bp] = 20 μM) a) without, and with b) [Ru(dip)$_2$dppz]$^{2+}$, c) [Ru(bpy)$_2$dppz]$^{2+}$, d) [Ru(dip)$_3$]$^{2+}$, and e) [Ru(dip)$_2$phen]$^{2+}$ ([Ru] = 10 μM) at 25 °C in a buffered solution (25 mM NaCl, 10 mM Tris-HCl, pH 7.5). bpy = 2,2'-bipyridine, phen = 1,10-phenanthroline.

$A_{24}T_{24}$, and $(GC)_6$ (Figure 3). However, compared with $[Ru(dip)_2dppz]^{2+}$, the use of these complexes did not result in strong negative signals around 290 nm, and $[Ru(bpy)_2dppz]^{2+}$, $[Ru(dip)_3]^{2+}$, and $[Ru(dip)_2phen]^{2+}$ are therefore not sufficiently powerful to induce conformational changes in B-DNA. When the structures of the complexes are considered, the tail-end ligand of $[Ru(dip)_2dppz]^{2+}$ is dip, which is much bigger than bpy in $[Ru(bpy)_2dppz]^{2+}$, and the intercalator ligand of $[Ru(dip)_2dppz]^{2+}$ is dppz, which is more powerful than dip in $[Ru(dip)_3]^{2+}$ and phen in $[Ru(dip)_2phen]^{2+}$. Thus, the specific structure of $[Ru(dip)_2dppz]^{2+}$ could be the origin for the conformational change from B-DNA to Z-DNA.

The characteristic signals of Z-DNA induced by $[Ru(dip)_2dppz]^{2+}$ (AT: 283 nm, GC: 290 nm) were shifted toward shorter wavelengths compared with previous reports (AT: 290 nm, GC: 293 nm).[33,39,40] We used 2D NOESY experiments to further confirm the formation of the Z conformation by full-AT, AT-tract, and full-GC sequences. The 2D NOESY experiments were performed on a Bruker AVANCE 500 MHz spectrometer at 298 K. The concentrations of $(AT)_{12}$, $A_{24}T_{24}$, and $(GC)_{12}$ were adjusted to 50 mM bp in 90% D_2O solution, and $[Ru(dip)_2dppz]^{2+}$ was added to the NMR samples to obtain a [Ru]/[bp] ratio of 0.1. Resolution of the full NOESY spectra of DNA during the conformational conversion induced by the binding of small molecules is complicated. Therefore, we selected spectral regions that showed a direct response to the B–Z transition.[41,42] Since the B–Z transition results from the conformational changes of A and G from *anti* to *syn*, AH8–AH1′ and GH8–GH1′ cross-peaks appear in the NOESY spectra (Figure 4).[43,44] NOESY experiments without the addition of $[Ru(dip)_2dppz]^{2+}$ were also carried out (Figure S4).

2D NOESY cross-peaks of H1′ and H3′ on deoxyribose and H8 on the base are a result of the B–Z transition and were observed in small selected regions. AH8–AH1′ and GH8–GH1′ cross-peaks were not observed in the absence of $[Ru(dip)_2dppz]^{2+}$ (Figure S4), thus indicating that no Z-DNA was formed. AH8–AH1′ and GH8–GH1′ cross-peaks were observed in the presence of $[Ru(dip)_2dppz]^{2+}$ (Figure 4), thus confirming that $(AT)_{12}$, $A_{24}T_{24}$, and $(GC)_{12}$ form Z-DNA. The AH8–AH3′ cross-peak was observed, even when only part of the DNA was converted into the Z form. We tried to use a mixing time as short as possible (50 ms) in the 2D NOESY experiments, because longer mixing times (usually > 300 ms) could lead to the appearance of unwanted signals. We also carried out 1D ^1H NMR analyses on a Bruker AVANCE 800 MHz spectrometer under similar conditions (Figure S5). Decreasing chemical shifts of AH3′ and AH1′ were observed as a result of the perturbation of AH8 by the B–Z transition.[45]

An electrophoretic mobility shift assay (EMSA) was performed to confirm the formation of $[Ru(dip)_2dppz]^{2+}$-induced Z-DNA. We used a Z-DNA antibody to verify the existence of Z-DNA in the $[Ru(dip)_2dppz]^{2+}$-treated DNA samples. Enzyme-digested pBR322 (lpBR322 with 4361 bp) was chosen for agarose gel electrophoresis as it contains a long strand (Figure 5 A).[46,47] The EMSA gel clearly showed that as the concentration of $[Ru(dip)_2dppz]^{2+}$ increased from 5 μM to 20 μM, the bands with the Z-DNA antibody (3.7 μg; Figure 5 A, lanes 3–5) ran slower than the bands without the Z-DNA antibody (Figure 5 A, lanes 6–8). This result implied the generation of a protein–DNA complex in the DNA sample. Thus, we were convinced that the formed Z-DNA led to the binding of lpBR322 to the Z-DNA antibody. Further-

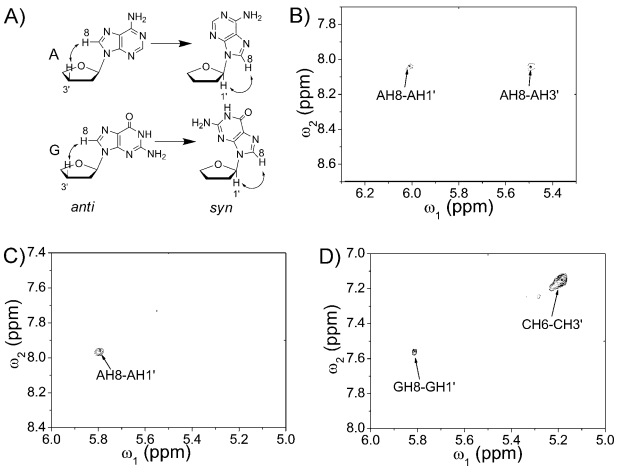

Figure 4. A) *anti* and *syn* conformations of A and G. Selected regions of 500 MHz 2D NOESY spectra of B) $(AT)_{12}$, C) $A_{24}T_{24}$, and D) $(GC)_{12}$ ([bp] = 50 mM) with $[Ru(dip)_2dppz]^{2+}$ ([Ru] = 5 mM) at 25 °C in a buffered solution (25 mM NaCl, 10 mM phosphate, pH 7.5). The conformational changes in the three sequences are confirmed by NOE cross-peaks between H8 of the base, and H1′ and H3′ of the deoxyribose.

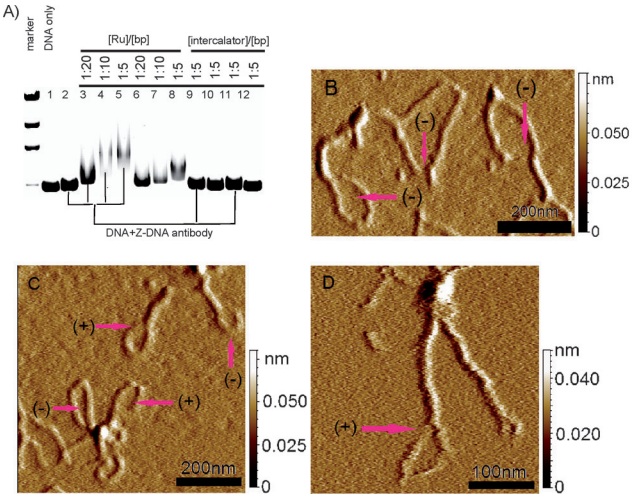

Figure 5. A) Agarose gel electrophoresis of lpBR322 (enzyme-digested linear pBR322 DNA, [bp] = 100 μM) treated with different additives and Z-DNA antibody in TAE buffer (40 mM Tris-HAc, 2 mM EDTA). Lane 1: DNA only, lane 2: DNA + Z-DNA antibody, lanes 3–5: DNA + $[Ru(dip)_2dppz]^{2+}$ + Z-DNA antibody, lanes 6–8: DNA + $[Ru(dip)_2dppz]^{2+}$, lane 9: DNA + $[Ru(dip)_3]^{2+}$ + Z-DNA antibody, lane 10: DNA + $[Ru(dip)_3]^{2+}$, lane 11: DNA + dppz + Z-DNA antibody, lane 12: DNA + dppz. B–D) AFM scans of closed-circle pBR322 ([bp] = 1 μM) on a mica substrate in air. Negatively supercoiled pBR322 (−) B) without $[Ru(dip)_2dppz]^{2+}$, C) with 0.1 μM $[Ru(dip)_2dppz]^{2+}$ ([Ru]/[bp] = 1:10), and D) with 0.2 μM $[Ru(dip)_2dppz]^{2+}$ ([Ru]/[bp] = 1:5). Positively supercoiled pBR322s = (+).

more, the samples without the Z-DNA antibody also showed a gradient with increasing $[Ru(dip)_2dppz]^{2+}$ dosage (Figure 5A, lanes 6–8). These results again confirmed the conformational change in the DNA, as it is well known that a strand in a Z-DNA conformation is longer and more rigid than the same strand in a B-DNA conformation and thus shows a different gel mobility. In addition, no reduction in band migration speed was observed when the Z-DNA antibody (3.7 μg; Figure 5A, lanes 9,11) or dppz as DNA base intercalator (Figure 5A, lanes 11,12) were added. These results excluded the influence of nonspecific binding between lpBR322 and the Z-DNA antibody, and also the influence of DNA elongation caused by base intercalators. We also excluded the influence of the positive charge of $[Ru(dip)_2dppz]^{2+}$ on the electrophoretic mobility by using AT and GC sequences (24 bp and 98 bp) to replace lpBR322 in the polyacrylamide gel electrophoresis, which gives a much higher resolution compared with agarose gel electrophoresis (data not shown). An electrophoretic mobility delay was not observed, which further indicated that the delay of lpBR322 in agarose gel electrophoresis resulted from the conformational change.

Closed-circle pBR322 is a plasmid-containing negatively supercoiled structure, which can stabilize a small segment of Z-DNA. The negatively supercoiled structures were clearly observed by AFM (Figure 5B, labeled by (−)). These negatively supercoiled structures release excess energy that results from the underwinding of B-DNA.[48] Under physiological conditions, the negatively supercoiled structure of the plasmid and the Z-DNA segments maintain a tautomeric equilibrium. Studies have shown that if one turn of the DNA helix changed from right-handed to left-handed, two negative supercoils disappear.[30] Therefore, we hypothesized that positively supercoiled structures would be observed if enough Z-DNA segments were present. The positively supercoiled structures induced by $[Ru(dip)_2dppz]^{2+}$ were clearly observed (Figure 5C,D, labeled by (+)). In Figure 5C, both negatively and positively supercoiled structures were identified, thus indicating a B + Z conformation of the plasmid DNA. These results also indicated the formation of excess Z-DNA segments in pBR322,[49] which were induced by $[Ru(dip)_2dppz]^{2+}$.

The transition kinetics of the sequences used in this work were measured by time-dependent CD spectroscopy at 250 nm and 285 nm. Monitoring the CD signals at 250 nm and 285 nm indicated the depletion of B-DNA and the generation of Z-DNA, respectively (see Figures 1C,D and 2B, and Figure S3). All the depleted and newly generated signals after the addition of $[Ru(dip)_2dppz]^{2+}$ corresponded to the Exponential Decay2 (ExpDec2)[50] function, which meant the transition process was a first-order reaction. The conversion was too fast to be detected for poly-d(AT) and poly-d(GC) and is thus likely to be a zero-order reaction. According to real-time CD spectra, the transition of full-AT sequences was faster than that of full-GC sequences with the same number of base pairs, because full-AT equilibrated earlier than full-GC. Longer double strands, including genomic DNA, poly d(AT), and poly d(GC), were also more easily converted into Z-DNA.

The binding affinity of the DNA-intercalating dppz ligand with various sequences is high.[51] We investigated binding of $(AT)_{12}$, $(GC)_{12}$, and $A_{24}T_{24}$ to $[Ru(dip)_2dppz]^{2+}$ by using isothermal titration calorimetry (ITC; Figure 6).[52,53] The binding of $[Ru(dip)_2dppz]^{2+}$ with $(AT)_{12}$, $(GC)_{12}$, $A_{24}T_{24}$, and

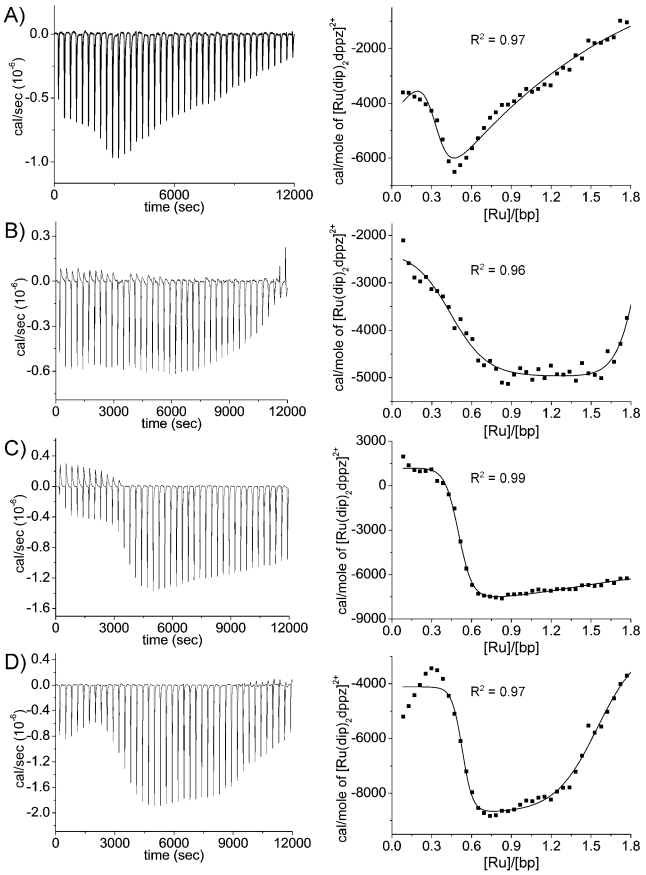

Figure 6. ITC titrations of A) $(AT)_{12}$, B) $(GC)_{12}$, C) $A_{24}T_{24}$, and D) pBR322 ([bp] = 100 μM, 1.4 mL) with $[Ru(dip)_2dppz]^{2+}$ ([Ru] = 1 mM, 240 μL) at 25 °C in buffered solutions (25 mM NaCl, 10 mM Tris-HCl, pH 7.5) with 10% DMSO. Raw calorimetric titration data (6 μL/injection) are shown on the left-hand side, and integrated data after subtraction of dilution heats and the fitting curves are shown on the right-hand side.

pBR322 induced the transition from the B to the Z conformation, and except for $A_{24}T_{24}$ at a ratio of [Ru]/[bp] < 0.5, these processes are exothermic. All integrated curves fit well to a biphasic model,[54,55] thus implying that there are two main processes in the titrations. Binding and B–Z transitions occur at [Ru]/[bp] ratios less than 0.5 ($(AT)_{12}$), 0.9 ($(GC)_{12}$), 0.6 ($A_{24}T_{24}$), and 0.6 (pBR322), at higher [Ru]/[bp] ratios mainly Z-DNA binding occurs. These ITC curves were not typical and further demonstrated the different binding interactions of $[Ru(dip)_2dppz]^{2+}$ with B-DNA and Z-DNA.

Further experiments were carried out to evaluate the possibility of Z-DNA formation induced by $[Ru(dip)_2dppz]^{2+}$ in living cells.[56] HeLa cells were incubated with $[Ru(dip)_2dppz]^{2+}$, and the CD spectrum showed a significant Cotton effect at 287 nm (Figure 7). The negative signal at 287 nm strongly indicated the formation of Z-DNA in living

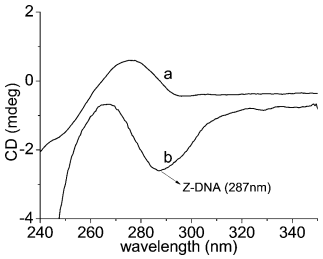

Figure 7. CD spectrum of HeLa cells incubated a) without and b) with $[Ru(dip)_2dppz]^{2+}$. HeLa cells were adjusted to a density of 10000 cm^{-2} and incubated for 48 h in neat Dulbecco's Modified Eagle's Medium (DMEM), then $[Ru(dip)_2dppz]^{2+}$ (500 μM) was added. After 2 h of incubation, the cells were washed three times with PBS buffer, collected, and monitored by CD spectroscopy.

cells, which is consistent with the transition of genomic DNA from the B to the Z conformation as outlined above. This interesting phenomenon means that $[Ru(dip)_2dppz]^{2+}$ may be used in in vivo experiments to induce the formation of Z-DNA, and thus cause a great change in biological processes of cells.

In conclusion, the ruthenium complex $[Ru(dip)_2dppz]^{2+}$ can induce the transition from B to Z DNA of various DNA sequences, which include non-APP and AT-rich segments, in a low-salt solution. The efficient transition to the Z-DNA conformation was observed by CD spectroscopy, NOESY experiments, gel electrophoresis, and AFM. In addition, we monitored the thermodynamics and kinetics of this transition by using ITC and real-time CD spectroscopy. Furthermore, we monitored the CD signal of Z-DNA in living HeLa cells. Because alternating AT sequences and poly-(dA)–poly-(dT) tracts are common and often found in the upstream direction of genes, it has been suggested that the transformation of these segments from the B to the Z conformation can regulate gene transcription. $[Ru(dip)_2dppz]^{2+}$ may thus be valuable for the in vitro and in vivo study of AT-rich Z-DNA. Notably, Z-DNA can be induced in high-salt conditions, thus enabling the stabilization of the left-handed double-helix conformation.

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