

Duplex Formation of the Simplified Nucleic Acid GNA

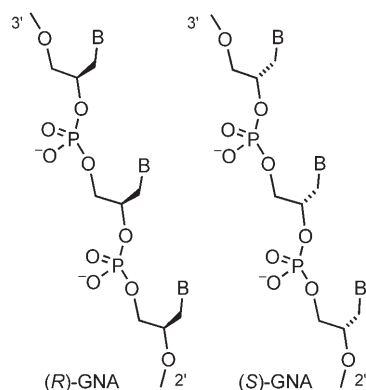
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Glycol nucleic acid (GNA) has an acyclic backbone of propylene glycol nucleosides that are connected by phosphodiester bonds. This paper characterizes the duplex-formation properties of this simplified nucleic acid. Although single and multiple GNA nucleotides are highly destabilizing if incorporated into DNA duplexes, the two enantiomeric oligomers (S)-GNA and (R)-GNA form antiparallel homoduplexes that are thermally and thermodynamically significantly more stable than analogous duplexes of DNA and RNA. The salt-dependence and Watson–Crick-pairing fidelity of GNA duplexes are similar to those of DNA duplexes, but, apparently, the 2'-deoxyribonucleotide and the propylene glycol backbones are not compatible with each other. This conclusion is fur-

ther supported by cross-pairing experiments. Accordingly, both (S)- and (R)-GNA strands do not generally pair with DNA. However, (S)-GNA, but not (R)-GNA, forms stable heteroduplexes with RNA in sequences that are low in G:C content. Altogether, the high stability and fidelity of GNA duplex formation in combination with the economical accessibility of propylene glycol building blocks for oligonucleotide synthesis render GNA an attractive candidate for the design of self-assembling materials. They further suggest that GNA could be considered as a potential candidate for a predecessor of RNA during the evolution of life on Earth.

Introduction

The DNA double helix is the target of extensive chemical modifications with the aim of understanding and altering its properties.^[1–7] We recently succeeded in demonstrating that a glycol nucleic acid (GNA) with a stripped-down acyclic backbone can form stable duplexes.^[8,9] The propylene glycol nucleotide building blocks contain just three carbons and one stereocenter, and are connected by phosphodiester bonds, thus yielding the two enantiomers (S)- and (R)-GNA.^[10] (S)- and (R)-GNA form



antiparallel duplexes with high thermal stabilities that significantly surpass the stabilities of analogous duplexes of DNA and RNA.^[8,9] Thus, with this constitution, GNA could display the most atom-economical solution for a functional nucleic acid backbone.^[11,12]

A very attractive feature of GNA is its straightforward chemical synthesis. Whereas the synthesis of base-modified deoxyri-

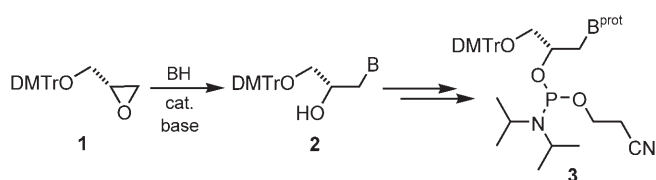
bose nucleotides generally includes a long reaction sequence and the painstaking separation of anomeric mixtures, this is not the case for GNA. Propylene glycol nucleotides^[13–17] with natural or artificial bases for automated solid-phase nucleic acid synthesis can conveniently be obtained in a few steps by regioselective and stereospecific epoxide ring-opening of protected or unprotected glycidols (e.g., **1**→**2**→**3**, Scheme 1).^[8,9,13a,c,15,16] GNA is therefore a particularly useful scaffold for designing artificial duplexes with altered chemical and physical properties in a highly economical fashion.

We provide here a progress report on the duplex-formation properties of GNA and compare its properties with those of DNA.

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Scheme 1. General synthesis of propylene glycol nucleotides by regioselective and stereospecific ring-opening of glycidol or glycidol derivatives with nucleophiles (1→2), followed by phosphoramidite (3) formation for automated oligonucleotide synthesis.

Results and Discussion

From glycol nucleotides in DNA to a completely acyclic GNA

The astonishing duplex stability of the acyclic GNA backbone made us wonder why this behavior had not been discovered earlier, especially given that acyclic nucleotides have been subject of intense interest in the past. Experiments within the last two decades revealed that adding one or more acyclic monomers to oligodeoxynucleotides resulted in strong thermal destabilization of the duplex structures.^[14,18] In order to understand the relationship between singly incorporated glycol nucleotides in DNA and completely acyclic GNA strands, we investigated different DNA/GNA hybrid systems by temperature-dependent UV spectroscopy at 260 nm as shown in Table 1.

Table 1. Thermal stabilities of duplexes of GNA, DNA, and DNA/GNA hybrids. ^[a] GNA nucleotides are highlighted in bold.		
Duplexes	T_m [°C] ^[b]	
1	5'-CACATTATTGTTGTA-3' 3'-GTGTAATAACAACAT-5'	47
2	5'-CACATTATTGTTGTA-3' 3'-GTGTAATAACAACAT-5'	34 (40)
3	5'-CACATT TTT GTTGTA-3' 3'-GTGTAATAACAACAT-5'	29 (28)
4	3'- CACATTATTGTTGTA -2' 3'-GTGTAATAACAACAT-5'	no T_m (no T_m)
5	5'-CACATTATTGTTGTA-3' 3'-GTGTAATAACAACAT-5'	33 (33)
6	5'-CACATT TTT GTTGTA-3' 3'-GTGTAATAACAACAT-5'	39 (39)
7	3'- CACATTATTGTTGTA -2' 2'-GTGTAATAACAACAT-3'	71

[a] Measured in 10 mM sodium phosphate (pH 7.0) with 100 mM NaCl and 2 μ M of each strand. [b] Shown for *S* nucleotides, the values for *R* nucleotides are given in brackets.

First, we substituted a deoxythymidine at position 8 in a 15-mer DNA duplex ($T_m = 47^\circ\text{C}$, Table 1, entry 1) for the glycol thymine nucleotides (*S*)-T and (*R*)-T. This single change resulted in a significant reduction in duplex stability to 34°C ($\Delta T_m = -13^\circ\text{C}$) and 40°C ($\Delta T_m = -7^\circ\text{C}$, entry 2), respectively. The incorporation of three glycol nucleotides into the 15-mer duplex DNA further decreases the stability of the duplex to 29°C

($\Delta T_m = -18^\circ\text{C}$) for the (*S*)-glycol nucleotides, and 28°C for the (*R*)-glycol nucleotides ($\Delta T_m = -19^\circ\text{C}$, entry 3). Moreover, having all 15 nucleotides in one DNA strand substituted for all-(*S*) or all-(*R*) glycol nucleotides completely destroys the helix formation (entry 4). Thus, single or multiple (*R*)- or (*S*)-glycol nucleotides incorporated into a DNA duplex strongly compromise the thermal duplex stabilities. These results are consistent with a report from Wengel's group more than a decade ago in which 17-mer oligonucleotides containing one or two (*S*)-1-(2,3-dihydroxypropyl)thymine nucleotides in middle positions have greatly reduced duplex stability.^[14,19]

We next substituted base pairs in DNA with glycol nucleotides. Entry 5 in Table 1 demonstrates that, in the case of a single GNA base pair, the stability is still strongly reduced by 14°C compared to that of the DNA strand. However, this reflects a destabilization of only 7°C per glycol nucleotide. Furthermore, replacing three consecutive DNA base pairs by base pairs containing the glycol backbone (entry 6) leads to a duplex that is now only destabilized by 8°C overall, or less than 1.3°C per acyclic nucleotide. Finally, replacing all the deoxyribose nucleotides of the 15-mer by (*S*)-glycol nucleotides provides an extremely stable duplex with a melting point of 71°C , which exceeds the stability of the analogous DNA duplex by 24°C (entry 7).

For the last decade it has been widely assumed that nucleic acid analogues containing a phosphodiester backbone need to be cyclic in order to produce the required conformational preorganization for duplex formation.^[14,18,20] This conclusion resulted, at least in part, from the disappointing duplex stabilities of DNA with incorporated single or multiple acyclic nucleotides.^[14,18,19] Extrapolation of these data implied that completely acyclic nucleic acid backbones would have no chance of stable duplex formation, again because of the missing conformational preorganization of the single strands for duplex formation. Table 1 and previous results from our group^[8,9] demonstrate that this assumption is not valid for the GNA backbone. Rather, Table 1 suggests that the destabilization of propylene glycol nucleotides in DNA duplexes is caused by a structural incompatibility of the 2'-deoxyribonucleotide and the glycol oligonucleotide backbone. Neither the (*S*)-GNA nor (*R*)-GNA enantiomer pairs properly with DNA.^[21] This observation might hold true for other acyclic systems as well, and a reinvestigation of completely artificial backbones with different acyclic nucleotides could lead to more surprising results.

Thermal and thermodynamic stabilities of GNA duplexes

Over the last two years we have investigated multiple GNA duplexes of different length and sequence. Table 2 summarizes the (*S*)-GNA strands that we analyzed for duplex formation by temperature-dependent UV spectroscopy at 260 nm. Except for hexamer sequences (e.g., Table 2, entry 1), self-complementary strands (entries 2–5) or 1:1 mixtures of complementary strands in an antiparallel fashion (entries 6–13) yield characteristic sigmoidal melting curves with melting points between 25 and 73°C . These data demonstrate that GNA is capable of antiparallel duplex formation with Watson–Crick base pairing in a

Table 2. Thermal and thermodynamic stabilities of (S)-GNA and DNA duplexes.^[a]

	Strand size	[NaCl]	Duplexes	T_m [°C] ^[b]	ΔG (298 K) [kcal mol ⁻¹] ^[c]
1	hexamer	1 M	3'-ATTAAT-2' 2'-TAATTA-3'	< 20	n.d.
2	octamer	1 M	3'-AAATATTT-2' 2'-TTTATAAA-3'	25	n.d.
3	octamer	150 mM	3'-AACTAGTT-2' 2'-TTGATCAA-3'	38	n.d.
4	octamer	500 mM	3'-CGAATTCG-2' 2'-GCTTAAGC-3'	54 (36)	-11.8 (-8.7)
5	octamer	150 mM	3'-ATGCGCAT-2' 2'-TACGCGTA-3'	57	n.d.
6 ^[9]	15-mer	100 mM	3'-CACATTATTGTTGTA-2' 2'-GTGTAATAACAACAT-3'	71 (47)	-22.4 (-13.5)
7	15-mer	500 mM	3'-AATATTATTATTTA-2' 2'-TTATAATAATAAAT-3'	59 (41)	-9.9 (-4.2)
8	15-mer	150 mM	3'-A ₁₅ -2' 2'-T ₁₅ -3'	65 (40)	-15.1 (-12.5)
9	15-mer	150 mM	3'-A ₇ CA ₇ -2' 2'-T ₇ GT ₇ -3'	64	n.d.
10	15-mer	150 mM	3'-A ₇ GA ₇ -2' 2'-T ₇ CT ₇ -3'	65	n.d.
11	15-mer	150 mM	3'-A ₆ C ₃ A ₆ -2' 2'-T ₆ G ₃ T ₆ -3'	73	n.d.
12 ^[8]	18-mer	200 mM	3'-TAAAATTTATATTATAA-2' 2'-ATTTTAAATATAAAT-3'	63 (41)	n.d.
13 ^[8]	18-mer	200 mM	3'-TTTTAAATTTTAAATATAT-2' 2'-AAAATTTAAATTATATA-3'	63	n.d.

[a] Measured in 10 mM sodium phosphate (pH 7.0), 2 μ M of each strand and the indicated NaCl concentrations. [b] Values for DNA are given in brackets. [c] ΔG values were estimated from van't Hoff plots by plotting T_m^{-1} against the natural logarithm of varying duplex concentrations. Each data point is the mean of three measurements.

general fashion. Furthermore, comparison of duplexes of GNA and DNA of the same sequence reveals that the acyclic backbone of GNA supports duplexes of significantly higher thermal stabilities, with melting points that are 18 to 25 °C higher for GNA than for DNA (entries 4, 6–8, and 12). This superior thermal stability goes along with an increased thermodynamic stability at room temperature (entries 4, 6–8)

GNA duplex melting analyzed by CD spectroscopy

Temperature-dependent CD spectroscopy with a mixed-sequence GNA duplex (Table 2, entry 6) confirms the formation of a thermally highly stable GNA duplex (Figure 1A). A strong Cotton effect with CD bands at around 205, 220, and 270 nm diminishes with increasing temperature. Quantitative analysis of the CD signal at 220 nm from 20 to 90 °C is shown in Figure 1B. The data can be fitted to a sigmoidal melting curve with an inflection point of 72 °C, which is in agreement with the melting point determined by UV-melting ($T_m = 71$ °C).^[22] Thus, both UV and CD spectroscopy support the cooperative duplex formation of complementary GNA single strands.^[23]

Fidelity of Watson–Crick base pairing in GNA

In order to verify that GNA forms duplexes that follow the Watson–Crick base-pairing rules, we examined the stability of a GNA duplex that included every possible base pair combination in the middle position of a mixed 15-mer sequence (Table 3). According to these results, GNA closely follows the Watson–Crick base pairing scheme with A:T and G:C being significantly more stable to any other base pair combination and with G:C base pairs being approximately 4 °C more stable than A:T base pairs. The thermal destabilization of mismatches in GNA duplexes ranges from $\Delta T_m = -6$ to -18 °C, compared to mismatches in DNA duplexes ranging from $\Delta T_m = -10$ to -23 °C (see bracketed values in Table 3). These data reveal that GNA discriminates strongly in favor of the Watson–Crick base-pairing scheme and only with slightly lower fidelity than DNA.

Salt-dependence of GNA duplex stability

In order to test the dependence of the ionic strength of the buffer on the thermal stability of the GNA duplex, we measured duplex melting points of a mixed GNA sequence (for the sequence, see Table 2, entry 6) as a function of changing NaCl concentrations. As shown in Figure 2, the thermal stability of the duplex GNA increases with increasing concentration of Na⁺. For example, raising the Na⁺ concentration from 60 to 810 mM leads to an increase in the duplex melting point by 13 °C. Under the same conditions, a DNA duplex of the same sequence increases the melting point by 14 °C. Thus, the salt dependence of the thermal stabilities of GNA duplexes is very closely related to that of DNA duplexes. Since the salt effect can be attributed to a modulation of the electrostatic repulsion between phosphodiester groups of opposite strands with varying concentrations of metal cations,^[24] we conclude that the distances between phosphodiester groups of opposite strands are very similar in GNA and DNA duplexes.

Cross-pairing of GNA with DNA and RNA

Finally, we further investigated the cross-pairing ability of GNA. We chose as a starting point the symmetrical sequence A₁₅:T₁₅

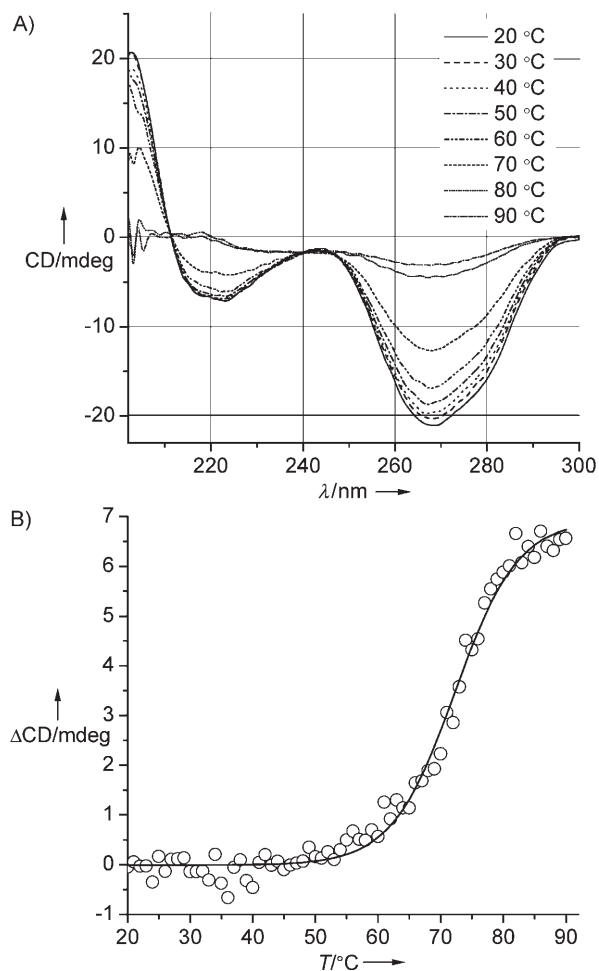


Figure 1. Temperature-dependent CD measurements with a GNA duplex (see Table 2, entry 6 for the sequence). A) CD spectra at different temperatures. B) Change in CD signal at 220 nm as a function of temperature, changed in 1 °C steps. Sigmoidal curve fitting yields a melting temperature of 72 °C, as calculated from the inflection point. Measured in 10 mM sodium phosphate (pH 7.0) with 100 mM NaCl and 2 μ M of each strand on a Jasco J-810 spectropolarimeter in a 5 mm path length quartz cuvette. Each spectrum (A) or data point (B) is the mean of five experiments.

Table 3. Thermal stabilities (T_m [°C]) of matched and mismatched Watson–Crick base pairs in duplex GNA. ^[a]					
3'-CACATTAXTGTGTA-2'					
2'-GTGTAATYACAAACAT-3'					
X					
	A	T	C	G	
A	65 (34)	71 (47)	63 (34)	65 (38)	
T	72 (47)	61 (36)	59 (34)	62 (35)	
C	63 (31)	61 (33)	58 (25)	75 (48)	
G	66 (36)	62 (36)	76 (48)	62 (38)	

[a] 10 mM sodium phosphate, 100 mM NaCl, 2 μ M duplex concentration. Results for DNA are given in brackets.

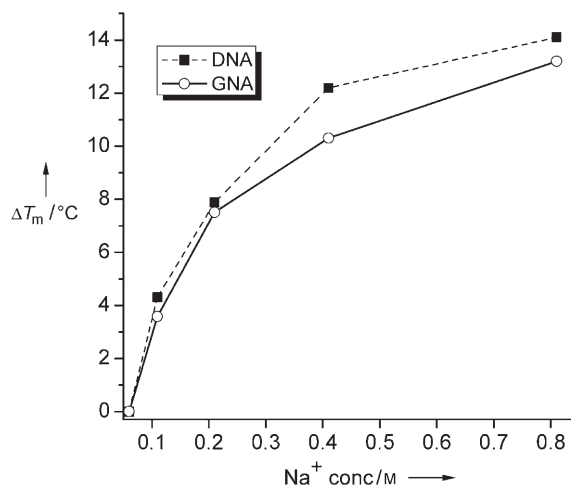


Figure 2. Comparison of the thermal stability of a GNA duplex with that of a DNA duplex of the same sequence (Table 2, entry 6; both 2 μ M each strand) as a function of Na⁺ concentration. Experiments were performed in 10 mM sodium phosphate (pH 7.0) with varying concentrations of NaCl. Each data point is the mean of two measurements.

in order to capture antiparallel as well as possible parallel duplex formations. The results of the cross-pairing of (*S*)- and (*R*)-GNA with each other, as well as with DNA and RNA, are shown in Table 4. The most stable duplexes are (*S*)-GNA:(*S*-

Table 4. T_m data [°C] for the cross-pairing between strands of (*S*)-GNA, (*R*)-GNA, RNA, and DNA.^[a]

	(<i>S</i>)-GNA A ₁₅	(<i>R</i>)-GNA A ₁₅	RNA A ₁₅	DNA A ₁₅
(<i>S</i>)-GNA T ₁₅	65	38	33	no T_m
(<i>R</i>)-GNA T ₁₅	35	64	no T_m	no T_m
RNA U ₁₅	28	no T_m	29	18
DNA T ₁₅	21	no T_m	35	40

[a] Conditions: 10 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 2 μ M of each strand.

GNA and (*R*)-GNA:(*R*)-GNA. A Job-plot analysis confirms a strand-pairing stoichiometry of 1:1 in this sequence context (see the Supporting Information). Cross-pairing between the two enantiomers is highly destabilized by $\Delta T_m = 26$ –30 °C. In fact, we could not detect cross-pairing between (*S*)- and (*R*)-GNA at all in previously published sequences.^[8]

The results in Table 4 further show that neither (*S*)- nor (*R*)-GNA significantly pairs with DNA. This is consistent with the data in Table 1 for mixed-sequence strands (entry 4) and previous results from our group.^[8] The UV-melting experiments imply that (*S*)-GNA but not (*R*)-GNA forms heteroduplexes with RNA that display similar stabilities to those of the analogous RNA duplex. These results are supported by CD spectroscopy. A CD spectrum of a 1:1 mixture of a T₁₅ (*S*)-GNA strand and an A₁₅ RNA strand is not just the sum of the individual single strands; this supports the formation of an (*S*)-GNA:RNA heteroduplex (Figure 3A). These RNA/GNA cross-pairing results are consistent with our earlier report in which we found that (*S*-

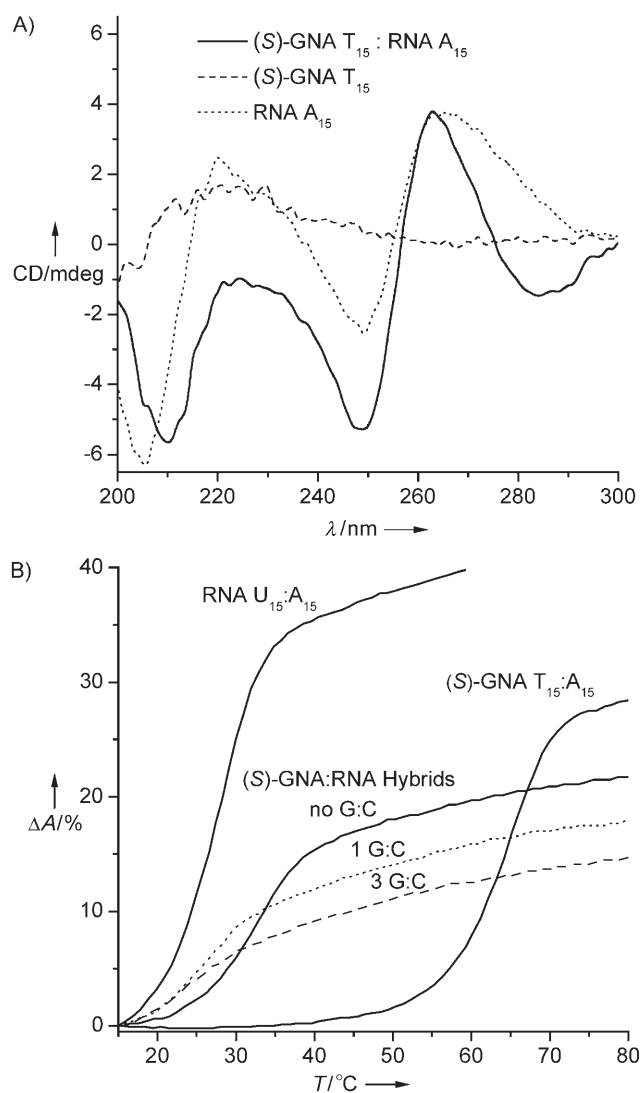


Figure 3. Cross-pairing of (S)-GNA with RNA. A) CD spectra of individual single strands (S)-GNA T_{15} and RNA A_{15} and of a 1:1 mixture thereof. Measured on an Aviv 62A DS spectrometer at 15 $^{\circ}\text{C}$ in a 1 mm path length quartz cuvette. Each spectrum is the mean of five measurements. Experiments were performed in 10 mM sodium phosphate (pH 7.0) with 1 mM EDTA and 150 mM NaCl and a concentration of each nucleic acid strand of 4 μM . B) UV-melting curves of 1:1 mixtures (2 μM each strand) of (S)-GNA A_{15} : T_{15} ($T_m = 65^{\circ}\text{C}$), RNA A_{15} : U_{15} ($T_m = 29^{\circ}\text{C}$), RNA:(S)-GNA A_{15} : T_{15} ($T_m = 33^{\circ}\text{C}$), RNA:(S)-GNA $A_7\text{CA}_7$: $T_7\text{GT}_7$ ($T_m = 24^{\circ}\text{C}$), and RNA:(S)-GNA $A_6\text{C}_3\text{A}_6$: $T_6\text{G}_3\text{T}_6$. Experiments were performed in 10 mM sodium phosphate (pH 7.0) with 150 mM NaCl and 1 mM EDTA.

GNA cross-pairs with RNA in an antiparallel fashion in an A:T-containing 18-mer sequence.^[8]

Surprisingly, when we next substituted one A:T base pair for G:C in the duplex sequence A_{15}/T_{15} to yield $A_7\text{CA}_7/T_7\text{GT}_7$, we found a strong decrease in the melting point ($\Delta T_m = 9^{\circ}\text{C}$) and hyperchromicity of the (S)-GNA:RNA heteroduplex (Figure 3B). Further increasing the number of G:C base pairs to three ($A_6\text{C}_3\text{A}_6/T_6\text{G}_3\text{T}_6$) diminished the hyperchromicity of the 1:1 (S)-GNA:RNA mixture to an even greater extent. In fact, this temperature-dependent UV curve is almost identical to the melting curve of an RNA single strand (see the Supporting Informa-

tion), and we therefore conclude that the three G:C base pairs prevent duplex formation between (S)-GNA and RNA in this sequence context. Moreover, a mixed 15-mer sequence (see Table 2, entry 6) that includes four G:C base pairs, did not show any cross-pairing between (S)-GNA and RNA (data not shown). It can be concluded that stable cross-pairing between (S)-GNA and RNA strands only occurs in strands that are predominately or entirely composed of A:T base pairs. The nature of the (S)-GNA/RNA heteroduplex might therefore differ from a canonical Watson–Crick duplex structure.

Conclusions

We have characterized the duplex formation of the acyclic nucleic acid GNA and compared it to DNA. Accordingly, temperature-dependent UV and CD spectroscopy demonstrate that GNA forms antiparallel duplexes with superior thermal and thermodynamic stabilities, whereas salt-dependence and base-pairing fidelity are very similar to DNA. Neither (S)-GNA nor (R)-GNA forms stable duplexes with DNA. This cross-pairing incompatibility of GNA with DNA is also reflected in the strong destabilization effect of glycol nucleotides in DNA. However, (S)-GNA, but not (R)-GNA, stably cross-pairs with RNA strands that contain only A:T base pairs. Surprisingly, G:C base pairs lead to a strong destabilization of these (S)-GNA/RNA hybrids. This study certainly raises many new questions. We are currently unraveling the reasons for the high duplex stability of GNA and the cross-pairing behavior of (S)-GNA with RNA.

Overall, the high fidelity of the Watson–Crick base pairing in GNA, in combination with the superior stability of GNA duplexes and the economical synthesis of the glycol nucleotide building blocks, renders GNA duplexes an exciting scaffold for designing self-assembling artificial materials with novel chemical and/or physical properties. Efforts to functionalize GNA are underway.

Experimental Section

GNA oligonucleotide synthesis and purification: GNA strands were prepared on an ABI Expedite Nucleic Acid Synthesizer or ABI 384 DNA/RNA Synthesizer. Oligonucleotides were synthesized on a 0.2, 0.5, or 1 μmol scale. A standard protocol for 2-cyanoethyl phosphoramidites (0.05 or 0.1 M) was used, except that the coupling of the phosphoramidites was extended to 3 min. After the trityl-on synthesis, the resin was incubated with concentrated aqueous ammonia at 55 $^{\circ}\text{C}$ for 12 h and then evaporated. The tritylated oligonucleotides were purified by C18 reversed-phase HPLC (Varian Dynamax 250 \times 10 mm, Microsorb 300–10, C18) with aqueous TEAA (0.05 M) and acetonitrile as the eluent. The oligonucleotides were then detritylated with 80% acetic acid for 20 min, precipitated with *i*PrOH after addition of sodium acetate, and again purified by HPLC. For this purification step, best purities were obtained with a Waters XTerra column (MS C18, 4.6 \times 50 mm, 2.5 μm) at 55–60 $^{\circ}\text{C}$ with aqueous triethylammonium acetate (TEAA; 0.05 M) and acetonitrile as the eluent. The identities of all oligonucleotides were confirmed by MALDI-TOF MS. DNA strands were purchased from Sigma Genosys or IDT (HPLC purified; Coralville, IA, USA) and RNA strands from IDT (HPLC purified). Oligonucleotide concentrations were determined by measuring the UV absorbance

of aqueous solutions. Extinction coefficients for DNA and RNA were used as provided by IDT (IDT OligoAnalyzer 3.0). Extinction coefficients for GNA (strands shown in 3'→2' direction) were calculated from deoxynucleotide increments and yielded the following ϵ_{260} values: ATTAAT: 65 070, AAATATTT: 86 760, AACTAGTT: 82 080, CGAATTCG: 77 400, ATGCGCAT: 77 400, AATATTATTATTTA: 153 630, TAAAATAATAATATT: 171 720, CACATTATTGTTGTA: 144 270, TACAA-CAATAATGTG: 162 360, A₁₅: 207 900, T₁₅: 117 450, A₇CA₇: 200 700, T₇GT₇: 119 970, A₇GA₇: 204 390, T₇CT₇: 116 280, A₆C³A₆: 186 300, T₆G₃T₆: 125 010, TAAAATTTATATTATTA: 195 210, TTAATAATATAA-ATTTTA: 195 210, TTTTAAATTTTAAATATAT: 183 150, ATATATTTAAAT-TTAAAA: 207 270 M⁻¹cm⁻¹.

Temperature-dependent UV spectroscopy: The melting studies were carried out in 1 cm path length quartz cells (200 μ L sample solution covered by mineral oil) on a Beckman DU800 spectrophotometer equipped with a thermoprogrammer. Melting curves were monitored at 260 nm with a heating rate of 1 °C min⁻¹. Melting temperatures were calculated from the first derivatives of the heating curves. Experiments were performed at least in duplicate, and mean values were taken.

CD spectroscopy: The CD studies were conducted with a Jasco J-810 spectropolarimeter in a 5 mm path length quartz cuvette (Figure 1) and an Aviv 62A DS spectrometer in a 1 mm path length quartz cuvette (Figure 3). Each spectrum or data point was acquired multiple times, and mean values were taken.

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Keywords: acyclic backbone • DNA • duplex formation • GNA • nucleic acids • simplified backbone

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