DNA Structures

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DNA and RNA in Anhydrous Media: Duplex, Triplex, and G-Quadruplex Secondary Structures in a Deep Eutectic Solvent**

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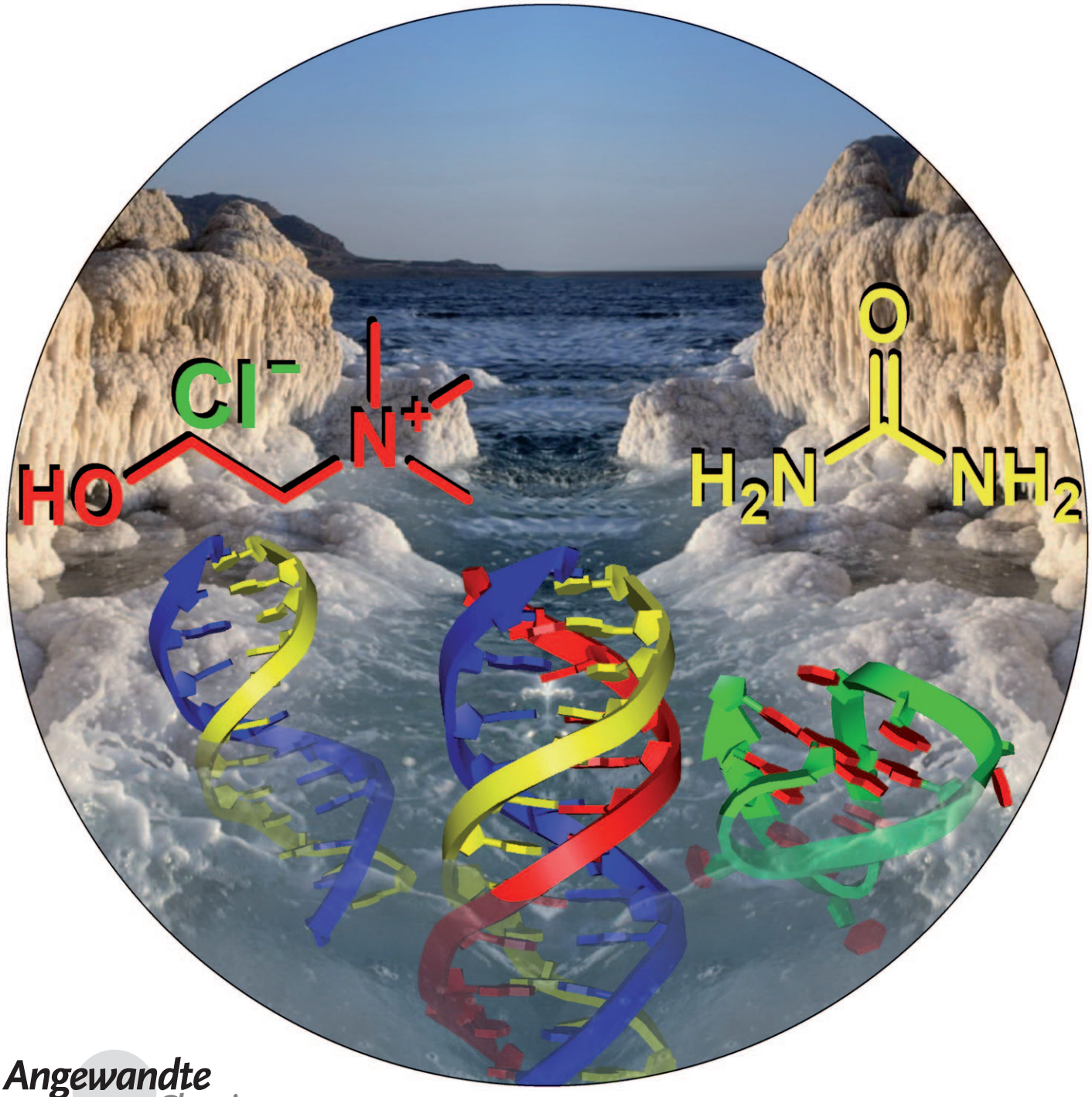

Room-temperature ionic liquids (RTILs) have generated tremendous interest as nonvolatile media that provide favorable environments for a wide range of chemical reactions.[1] A closely related class of solvents with physical properties and phase behaviors very similar to those of RTILs are room-temperature deep-eutectic solvents (DESs), which were developed by Abbott and co-workers.[2] These eutectic mixtures are attractive alternatives to RTILs, as DESs can be less expensive, more synthetically accessible, nontoxic, and biodegradable. Herein, we report that nucleic acids can form several secondary structures that reversibly denature with heating in a water-free DES. In some cases, the nucleic acid sequences studied exhibited different relative stabilities and different secondary structures in the DES to those in aqueous media. The results presented suggest that DESs and RTILs can be used as media for nucleic acid based technologies, and they have direct implications regarding the perceived necessity of water for nucleic acid secondary structure.[3]

The melting point of choline chloride (ChCl) is 302 °C, and that of urea is 133 °C, whereas a 1:2 ChCl/urea mixture melts at 12 °C.[2a] This DES was prepared for the present study by a solvent-free process; solid ChCl and urea were heated until the eutectic formed.[2a] Solutions of DNA and RNA in the DES were prepared by mixing aqueous stock nucleic acid solutions with the DES and then subjecting the mixture to vacuum centrifugation until a constant mass was reached (at least 12 h). Karl Fischer[4] analysis revealed that the DES contained less than 0.25 % water (see the Supporting Information).

Circular dichroism (CD) was used to monitor nucleic acid structure.[5] A 32 bp DNA duplex of mixed GC/AT sequence composition in a low-salt buffer (100 m*m* NaCl [*m* = molal], 10 m*m* sodium phosphate, pH 7) exhibited a CD spectrum consistent with a B-form duplex, as expected (Figure 1 a). In the DES, the positive band was both significantly more intense and blue-shifted (Figure 1 a). These differences indicated a change in secondary structure (see below). In the buffer, the duplex exhibited a cooperative, reversible melting transition with a midpoint (T_M) at 73 °C. In the DES, the cooperative, reversible transition was retained (see Figure S1 in the Supporting Information), but the T_M value was diminished to 37 °C.

The CD spectrum of the 32 bp DNA duplex in the DES is suggestive of an A-form helix.[5] Most mixed-sequence DNA duplexes will undergo a B-to-A-form helix transition when subjected to dehydrating conditions and high ionic strength,[6] which are certainly characteristic of the DES. An RNA duplex (which adopts an A-form helix, even in a low-salt aqueous solution) exhibited similar CD spectra in the DES and in various aqueous buffers, although the spectrum in the DES was red-shifted by 5 nm (Figure 1 b).

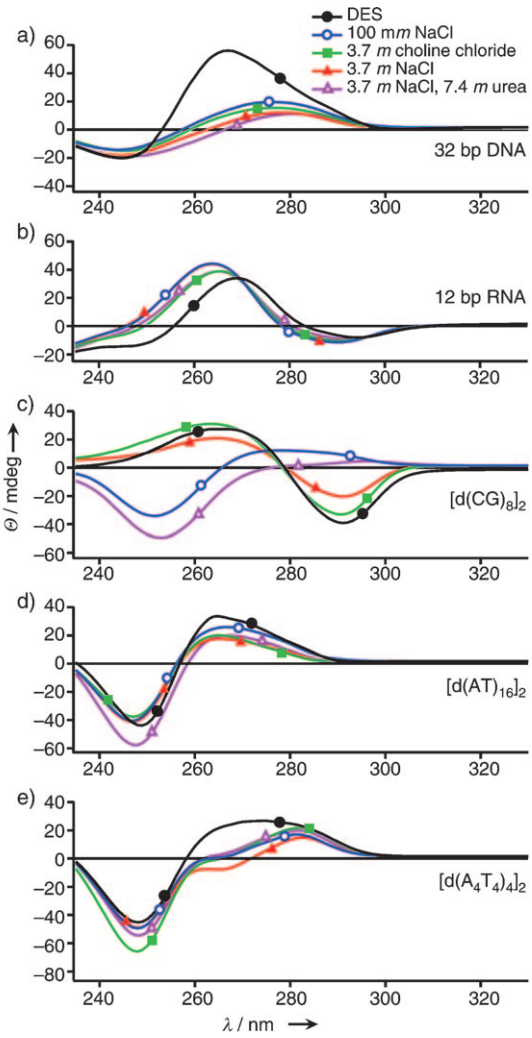

Figure 1. CD spectra of various DNA- and RNA-duplex samples. The concentration of all samples was 1.6 m*m* with respect to the nucleotides. Spectra were collected in cells with a path length of 1 mm. All aqueous solutions contained sodium phosphate (10 m*m*, pH 7). Spectra were acquired at 5 °C.

It is perhaps not surprising that duplex stability is lower in the DES than in an aqueous solution (Table 1), as urea is commonly used as a denaturant, and the DES contains urea (7.4 *m*) and ChCl (3.7 *m*). In an aqueous solution containing urea (7.4 *m*) and NaCl (3.7 *m*), the T_M value of the 32 bp DNA duplex was 65 °C; thus, both T_M values measured in aqueous solutions were significantly higher than the T_M value observed in the DES. In 3.7 *m* aqueous ChCl, the T_M value was 83 °C (Table 1). Thus, the depressed T_M value in the DES cannot be attributed entirely to either solvent component. Instead, it is the result of distinct solvent properties of the DES, the elimination of bulk water, or both. The T_M values observed for the RNA duplex support this conclusion (Table 1).

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Table 1: T_M values [°C] of DNA and RNA duplexes in various solvents.

Nucleic acid	NaCl (100 m*m*)[a]	DES	NaCl (3.7 *m*)[a]	ChCl (3.7 *m*)[a]	NaCl, urea[a,b]
32 bp DNA[c]	73	37	85	83	65
12 bp RNA	$\geq$95	44	>95	69	83
$d(CG)_8$	$\geq$85	44	n.d.[d]	72	76
$d(AT)_{16}$	57	29	68	77	38
$d(A_4T_4)_4$	57	26	73	79	52

[a] Aqueous solutions contained sodium phosphate (10 m*m*, pH 7). [b] NaCl (3.7 *m*), urea (7.4 *m*). [c] See the Supporting Information for nucleotide sequences. [d] Not determined owing to a broad melting transition.

Given the apparent preference of the mixed GC/AT DNA duplex for the A-form helix in the DES, we considered whether duplexes with CG repeats, which can exhibit a B-to-Z-form transition under high-salt or dehydrating conditions, might also exhibit an alternative structure in the DES. We selected the oligonucleotide $d(CG)_8$ for this investigation. The CD spectrum of $d(CG)_8$ in the DES was dramatically different from that observed in the low-salt buffer (Figure 1c). The inverted CD spectrum obtained in DES, with a negative band at 290 nm and a positive band at 260 nm, is indicative of a left-handed Z-form helix.[7] Similar spectra were observed in aqueous solutions containing ChCl (3.7 *m*) or NaCl (3.7 *m*), but not NaCl (3.7 *m*) and urea (7.4 *m*; Figure 1c). The spectra in 3.7 *m* aqueous ChCl and the DES are particularly similar, which strongly suggests that the helical structures of $d(CG)_8$ in these two environments, one aqueous and one anhydrous, are the same.

DNA polymers with AT repeats can also adopt helical structures that deviate from the canonical B form, including cation-dependent variants of the B- and A-form helices in solution and in fibers,[8] and the Z form at high ionic strength and elevated temperature.[9] We therefore examined the oligonucleotide $d(AT)_{16}$. Unlike the mixed-sequence 32 bp DNA or $d(CG)_8$, $d(AT)_{16}$ did not exhibit an appreciably different CD spectrum in the DES (Figure 1d). This result suggests that the helical structure is similar to that in the aqueous solutions.

The duplex formed by the sequence $d(A_4T_4)_4$ is also of interest, because it contains four A-tract sequence elements, which are defined as four or more A·T base pairs without a 5′-TpA-3′ step. These sequence elements are known to adopt an altered B-form helical structure, designated B*, with an unusually narrow minor groove and high base-pair propeller twist,[10] and a propensity for cation localization in the minor groove.[11] For $d(A_4T_4)_4$, any B*-form helical structure is predicted to be interspersed with B-form helical structure, as A tracts are disrupted by 5′-TpA-3′ steps.[10] The CD spectrum of $d(A_4T_4)_4$ in the low-salt buffer at 5 °C is consistent with a mixed B-/B*-form structure (Figure 1e), and spectra of the same general shape were observed for other aqueous buffer conditions. In the DES, however, $d(A_4T_4)_4$ exhibited a significantly different CD spectrum (Figure 1e). Although the origin of this spectral difference is not obvious, it may represent a significant change in secondary structure. Nevertheless, a cooperative transition at 26 °C indicates that $d(A_4T_4)_4$ does form a secondary structure in the DES (see Figure S2 in the Supporting Information).

Nucleic acids can also form triplex structures, in which a homopyrimidine strand forms Hoogsteen base pairs with the purine residues of a homopurine–homopyrimidine Watson–Crick duplex.[12] To investigate potential triplex formation within the DES, we prepared samples of $d(A)_{16}$ and $d(T)_{16}$ in a 1:2 molar ratio. Triplexes typically require divalent cations or high monovalent-cation concentrations to be stable at room temperature.[12d] A 1:2 mixture of $d(A)_{16}$ and $d(T)_{16}$ in a buffer with $MgCl_2$ (50 m*m*) exhibited a CD spectrum consistent with triplex formation (Figure 2a).[12c] This system exhibited a third-strand T_M value of 20 °C, and a T_M value of 44 °C for the $d(A)_{16}{\cdot}d(T)_{16}$ duplex (see Figure S3 in the Supporting Information). In the DES, the CD spectrum of this system was significantly different (Figure 2a). Furthermore, only one melting transition was observed, at 66 °C (see Figure S3 in the Supporting Information). At first, we thought these observations were an indication that the triplex was not stable in the DES. However, the addition of the spectra of samples containing 1:1 $d(A)_{16}{\cdot}d(T)_{16}$ and only $d(T)_{16}$ in the DES, at concentrations that would occur if the triplex did not form at all in the DES at 15 °C (i.e., if no interaction occurred between the duplex and the third strand), yielded a spectrum that differed from that of the putative triplex in the DES (see Figure S4 in the Supporting Information). Thus, the additional molar equivalent of the $d(T)_{16}$ strand appears to interact with the $d(A)_{16}{\cdot}d(T)_{16}$ duplex.

As an additional means to verify triplex formation in the DES, a Job plot was used to investigate the stoichiometry of the $d(T)_{16}$–$d(A)_{16}$ interaction (Figure 2b). The inflection observed in this plot at $x = 0.67$ supports triplex formation (i.e., a 1:2 $d(A)_{16}$–$d(T)_{16}$ complex). Surprisingly, but in agreement with the observed single melting transition, the Job plot analysis did not indicate the formation of a 1:1 complex between $d(A)_{16}$ and $d(T)_{16}$ in the DES. These results

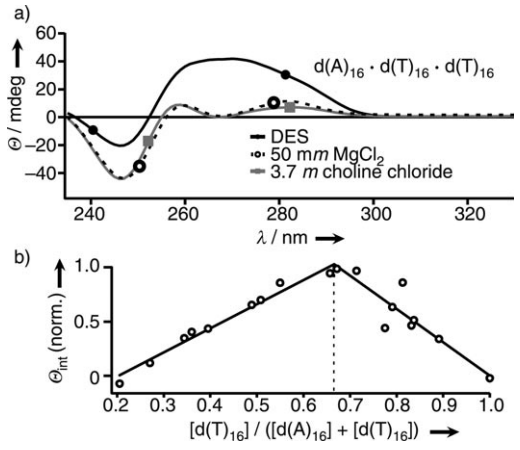

Figure 2. a) CD spectra of $d(A)_{16}$ and $d(T)_{16}$ in a 1:2 molar ratio, with a total nucleotide concentration of 1.6 m*m*. Aqueous buffers contained sodium phosphate (10 m*m*, pH 7). Spectra were acquired at 5 °C. b) Job plot analysis at 15 °C of mixtures of $d(T)_{16}$ and $d(A)_{16}$ with a constant nucleotide concentration of 1.6 m*m*. The *y* axis shows the integrated CD signal from 235–300 nm. The left line is the linear fit for $x = 0.2$–0.67; the right line is the linear fit for $x = 0.67$–1.0.

suggest that the $d(A)_{16}\cdot[d(T)_{16}]_2$ triplex is of equal or greater stability than the $d(A)_{16}\cdot d(T)_{16}$ duplex, an observation that is consistent with previous reports of enhanced triplex stability under conditions of high salt, molecular crowding, or low water activity.[12c, e–g]

In a buffer containing ChCl (3.7 *m*), the 1:2 $d(A)_{16}/d(T)_{16}$ mixture exhibited a spectrum very similar to that observed in the buffer containing $MgCl_2$ (50 m*m*; Figure 2a). The T_M value for the triplex in 3.7 *m* aqueous ChCl was 28 °C, and that for the duplex was 71 °C (see Figure S3 in the Supporting Information). These results indicate that aqueous choline, at high concentration, stabilizes a DNA triplex and a homopurine–homopyrimidine duplex.

G-quadruplex structures are currently of substantial interest in medicine, self-assembly, and nanotechnology.[13] In the present study, we used the thrombin-binding aptamer (TBA)[14] to test the stability of the G-quadruplex secondary structure in the DES. TBA, like other G-quadruplexes, is stabilized by the coordination of cations (e.g., Na^+ or K^+) in the center of G-tetrads. As expected, TBA did not form a stable G-quadruplex in the DES, as the only cation present in significant concentration is choline, which is too large for coordination by G-tetrads. However, the addition of KCl (100 m*m*) to the DES gave a spectrum similar to that observed for TBA in an aqueous buffer containing KCl (100 m*m*; Figure 3). As in the aqueous buffer, TBA exhibited a cooperative, reversible thermal transition in the DES with KCl (100 m*m*; see Figure S5 in the Supporting Information).

The stability of the TBA G-quadruplex in the DES with added cations was quite different from that in an aqueous buffer. With KCl (100 m*m*) present, the T_M value was 59 °C in DES, in comparison to 50 °C in an aqueous buffer. In contrast, the T_M value of TBA in an aqueous buffer was 20 °C if NaCl (100 m*m*) was present, whereas TBA did not form a stable G-quadruplex in the DES with NaCl (100 m*m*; Figure 3). Thus, the T_M value of the K^+ form of TBA *increased* by 9 °C in the DES relative to that in an aqueous buffer, and the T_M value of the Na^+ form of TBA *decreased* by at least 20 °C. Given that G-quadruplex stability is strongly coupled to the solvation free energy of the cation,[15] the greater stability difference between the Na^+ and K^+ forms of TBA in the DES could result from differing solvation free energies of Na^+ and K^+ in the DES and water.

Finally, as an initial investigation of nucleic acid duplex stability in an RTIL, we examined the 32 bp DNA duplex and $d(CG)_8$ in a popular ionic liquid, *N*-methylimidazolium tetrafluoroborate (HMIm BF_4). In HMIm BF_4, $d(CG)_8$ exhibited secondary-structure formation, whereas the 32 bp DNA duplex was at least partially denatured—despite the comparable stability of these duplexes in the DES (see Figure S6 in the Supporting Information). Neither duplex exhibited the apparent B-to-A or -Z transition observed in the DES. Like molecular solvents, specific DESs and RTILs appear to vary in their interactions with the secondary structure of the (bio)polymers they dissolve. The structural similarity of choline to betaine and tetraalkylammonium ions may explain some of the characteristics of the DES in this regard, as these ions have been reported to exhibit sequence-dependent modulation of DNA-duplex stability.[16]

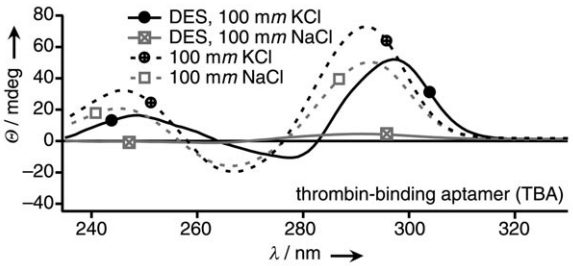

Figure 3. CD spectra of the thrombin-binding aptamer (TBA) in various solvents. Samples had a total nucleotide concentration of 1.6 m*m*. Aqueous solutions contained sodium phosphate (10 m*m*, pH 7). Spectra were recorded at 5 °C.

There have been earlier reports of DNA maintaining a duplex structure in nonaqueous solvents, but these studies were apparently limited to glycerol and ethylene glycol.[17] Previous studies of DNA structural integrity in RTILs were limited to hydrated ionic liquids.[1f] To the best of our knowledge, our results serve as the first demonstration that no fewer than four distinct nucleic acid structures can exist in DESs or RTILs. In *N*,*N*-dimethylformamide, mononucleotides were reported to polymerize by dehydration condensation.[18] Owing to their anhydrous character and capacity to support natively folded DNA and protein structures, DESs and RTILs are appealing media for the nonenzymatic synthesis of biopolymers. Additionally, given previous reports that these solvents can support enzyme catalysis, the possibility that catalytic nucleic acids and enzyme–nucleic acid complexes could be used in these solvents is enticing.

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