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Oligonucleotide Gas-Phase Hydrogen/Deuterium Exchange with D_2S in the Collision Cell of a Quadrupole-Fourier Transform Ion Cyclotron Resonance Mass Spectrometer

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We have implemented gas-phase hydrogen/deuterium exchange (HDX) experiments in the external collision cell of a hybrid quadrupole-Fourier transform ion cyclotron resonance mass spectrometer. In this configuration, multiply charged oligonucleotide anions undergo significant exchange with D_2S at reaction intervals ranging from 0.11 to 60.1 s. For DNA homohexamers, relative exchange rates were $dC_6 \sim dA_6 > dG_6 > dT_6$, correlating with the gas-phase acidities of nucleobases (C > A > T > G), except for guanine. Our results are consistent with a relay mechanism in which D_2S interacts with both a backbone phosphate group and a neutral nucleobase through hydrogen bonding. We propose that the faster exchange of polyguanosine compared to polythymidine is due to the larger size of guanine and the orientation of its labile hydrogens, which may result in gas-phase conformations more favorable for forming complexes with D_2S. Similar trends were observed for RNA homohexamers, although their HDX rates were faster than for DNA, suggesting they can also exchange via another relay process involving the 2′-hydroxyl group. HDX of DNA duplexes further supports the involvement of nucleobase hydrogens because duplexes exchanged slower than their corresponding single strands, presumably due to the intermolecular hydrogen bonds between nucleobases. This work constitutes the first investigation of the mechanisms of oligonucleotide gas-phase HDX. Our results on duplexes show promise for application of this strategy to the characterization of structured nucleic acids.

RNA hairpins represent a common nucleic acid structural motif with multiple biological functions, including definition of nucleation sites for folding,[1] determination of tertiary interactions in RNA enzymes,[2,3] protection of mRNAs from degradation,[4,5] recognition by RNA-binding proteins, involvement in mRNA localization,[6] and retroviral encapsidation and packaging.[7,8] Because only a few RNA structures have been solved, determination of hairpin motifs can provide valuable information for RNA folding and structural prediction.[9] Nucleic acid higher order structure (e.g., hairpins and intermolecular duplexes) is also of high importance in antisense and antigene strategies toward novel chemotherapeutic agents.[10−14] Several techniques exist for characterization of the noncovalent interactions involved in nucleic acid higher order structure, including NMR spectroscopy,[15,16] fluorescence resonance energy transfer (FRET) employing fluorescent labels,[17] and mass spectrometric approaches, such as ion mobility analysis[18] and combination with solution-phase chemical footprinting.[19] However, NMR requires large sample quantities and has an upper mass limit, FRET only provides distance constraints in a limited region of the molecule, ion mobility only provides a molecular cross section (thus, does not provide information on the overall structure), and chemical footprinting requires extensive sample manipulation prior to MS analysis. We have applied tandem mass spectrometry (MS^n) involving electron detachment dissociation (EDD[20]) to differentiate isomeric DNA 15-mers with different predicted structures.[21] However, that approach is currently limited to smaller molecules due to the low fragmentation efficiency of EDD.

Hydrogen/deuterium exchange (HDX) is a well-established technique for probing solution-phase structure of biological

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molecules based on the accessibility of various potentially exchangeable hydrogens.[22−25] However, due to the high opening rates of nucleobase pairs, HDX of structured nucleic acids in solution is very fast (~ms), rendering it difficult to track reaction details. Gas-phase HDX[26−30] in which rate constants are much smaller than those in solution has been proposed as an alternative method for structural analysis of nucleic acids.[29,31−37] This approach has become more feasible for larger nucleic acids due to the introduction of soft ionization methods, such as matrix-assisted laser desorption/ionization[38,39] and electrospray ionization (ESI).[40,41] Comparison of gas- and solution-phase conformations can provide insight into the nature of these ionization processes, particularly the role of solvent. Mass spectrometers are well suited for performing gas-phase HDX experiments because deuterium incorporation can be monitored directly from mass spectral peak shifts with reaction time, which is easily controlled by data acquisition programs. In particular, ion trapping techniques, such as ion cyclotron resonance (ICR) and quadrupole ion traps, can provide gas-phase HDX kinetics because ions can be trapped for extended time periods, allowing for observation of the relatively slow exchange processes.[29]

DePuy and Bierbaum suggested that HDX can be observed between anions and exchanging reagent that is as much as 20 kcal/mol less acidic.[42] Ausloos and Lias also stated that HDX was not observed for protonated compounds if the gas-phase basicities of the exchanging reagents differed by more than 20 kcal/mol.[43] However, HDX can still occur between reagents with large acidity differences, presumably due to complex formation.[26,33,44,45] Among the deuterated reagents explored for gas-phase HDX of nucleic acids, D_2S is most suitable[37, 44] because its pK_a is closer to the deprotonated backbone phosphate groups than those of other reagents, such as ND_3.[43,45] Consequently, HDX rate constants were found to be much higher for D_2S than for D_2O (minutes vs hours at 5×10^{-8} Torr pressure) due to the smaller gas-phase acidity difference between D_2S and nucleic acids (>20 vs > 40 kcal/mol for D_2O).[44] Furthermore, D_2S can very easily be introduced into a mass spectrometer because it is a gas at room temperature. Most previous HDX studies of nucleic acids involved mono(deoxy)nucleotides with a few exceptions in which 5- and 20-mer phosphorothioate oligodeoxynucleotides were characterized.[34,35,46] However, the exchange mechanism has only been discussed for mononucleotide phosphates. A relay process involving a 5′-phosphate group and the 3′-hydroxyl group of the sugar but not the nucleobase or the 2′-hydroxyl group has been proposed for exchange of 5′-monophosphate anions with D_2S.[37] By contrast, the 2′-hydroxyl group has been proposed to play an important role in gas-phase HDX of mononucleotide cations.[45] Here, we attempt to elucidate the role of nucleobases in the gas-phase HDX process of nucleic acid anions. Furthermore, in order to explore the analytical utility of this approach, we applied gas-phase HDX with D_2S to DNA duplexes to determine whether their exchangeable nucleobase hydrogens involved in base pairing would be protected from exchange and thereby display slower exchange rates. In a previous study, a quadruplex was shown to undergo more rapid HDX than its constituent monomer,[36] a behavior that was not expected. However, quadruplexes do not contain Watson−Crick base pairs.

EXPERIMENTAL SECTION

Sample Preparation. DNA and RNA hexamers, including dT_6, dC_6, dA_6, dGCATAC, dGCATGC, dTGGGGT, A_6, U_6, and 2′-methylated (2′-OMet) RNA were purchased from either TriLink Biotechnologies (San Diego, CA) or the Yale Keck Facility (New Haven, CT). All reagents were used without further purification. Oligonucleotides were diluted into 5% v/v 2-propanol (Fisher, Fair Lawn, NJ) with 10 mM ammonium acetate (Fisher) to a concentration of 10−100 μM (adjusted to yield a signal-to-noise ratio of 10 in 8 scans). Duplex formation was promoted by annealing DNA at 95 °C for 5 min, cooling down to 70 °C for 30 min, and storing overnight at 4 °C.

Mass Spectrometry. All experiments were conducted on an actively shielded 7-T quadrupole-Fourier transform ion cyclotron resonance (Q-FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA) in negative ion mode as previously described.[21] Briefly, oligonucleotide solutions were infused via an external Apollo II ion funnel electrospray ion source at a flow rate of 50 μL/h with the assistance of N_2 nebulizing gas. The inlet capillary was set to 2.8 kV for generation of oligonucleotide anions. N_2 drying gas (120 °C, 3.9 L/s) was applied to assist ESI droplet desolvation. HDX data were generated from doubly deprotonated precursor ions for DNA or RNA hexamers and triply deprotonated ions for DNA duplexes. The entire precursor isotopic envelope was quadrupole selected and stored in a hexapole collision cell in the presence of D_2S for 0.11−60.1 s. Multiple ICR cell fills were

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not used to avoid generation of mixtures of ion populations with different exposure time to D_2S (there is residual D_2S outside the storage hexapole). D_2S (Cambridge Isotope Laboratories, Andover, MA) was leaked into the collision cell to a pressure of $\sim 5 \times 10^{-6}$ mbar (gauge factory calibrated for nitrogen; no additional calibration was performed). Although different gas-phase conformations of nucleic acids may exist, hydrogens residing on different positions may exchange at different rates. Percentage of HDX was calculated based on the average m/z of the entire oligonucleotide isotopic distribution, i.e., by considering the relative abundance of each isotopic peak, according to the following equation (adapted from Zhang and Smith[47])

$$\% \text{ deuterium incorporation} = [(m/z)_{obs} - (m/z)_0]/[(m/z)_{max} - (m/z)_0] \times 100\%$$

in which $(m/z)_{obs}$ is the observed average m/z following a particular exchange time, $(m/z)_0$ the average m/z prior to HDX, and $(m/z)_{max}$ the expected average m/z at full deuteration of all exchangeable hydrogens. Error bars (higher errors are expected with more deuterium incorporated) were generated from data (three repeats) acquired on the same day due to difficulties with reproducing the D_2S pressure in the collision cell.

RESULTS AND DISCUSSION

Number of Exchangeable Hydrogens. The HDX process of oligonucleotides requires a deuterium acceptor group and a hydrogen donor group.[33,44] For both DNA and RNA, the most acidic sites are the backbone phosphate groups,[44,48] which are deprotonated in negative ion mode and therefore constitute possible deuterium acceptors during HDX. Each phosphate group (excluding the deprotonated ones) has one exchangeable hydrogen while nucleobases have varied numbers: adenine and cytosine both have two exchangeable hydrogens, guanine has three, and thymine and uracil both have one. In addition, the two end hydroxyl groups in DNA and RNA each have one exchangeable hydrogen. Therefore, the total numbers of exchangeable hydrogens for the doubly deprotonated DNA hexamer anions are 17 for dA_6, dC_6, and dGCATAC, 19 for dTGGGGT, 11 for dT_6, and 18 for dGCATGC. For RNA, the 2′-hydroxyl group of each sugar adds additional exchangeable hydrogens. Therefore, the doubly deprotonated RNA hexamers have six more exchangeable hydrogens than their corresponding DNA hexamers.

Gas-Phase Acidity of Nucleic Acids. It is generally believed that HDX is more favorable if the gas-phase acidities of the analyte and the deuterated reagent are close in value. Calculated acidities of the deuterium donor (D_2S) and acceptor (phosphate diester bridge in nucleic acids) are 351.3 and 329.0 kcal/mol, respectively.[44,48,49] Their difference is more than 20 kcal/mol, which is energetically unfavorable for HDX. However, previous experiments showed that exchange can still be observed between reagents with large acidity differences, which was attributed to complex formation between reagents, followed by a relay exchange process.[28,37,44,45] Prior experimental determination of the gas-phase acidities of nucleobases yielded the following trend, C > A > T > G, all with lower values than that of D_2S; thus, acidity differences between nucleobases and D_2S follow the trend C < A < T < G. Chan and Enke found that reaction efficiency increased as the gas-phase acidity difference between anions and neutral reagent decreased.[50] Therefore, the order of DNA HDX rates should hypothetically follow the acidity trend, C > A > T > G.

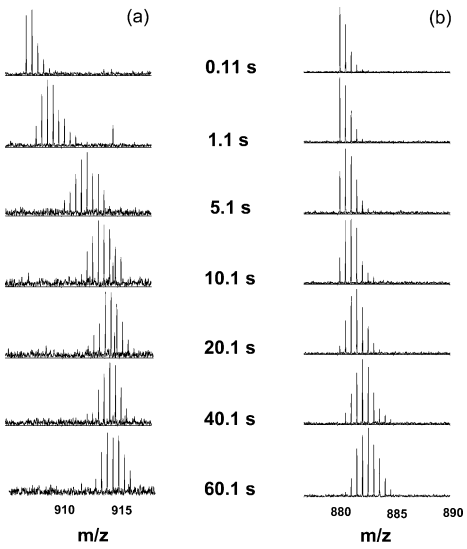

Figure 1. *m/z* shift of doubly deprotonated DNA following different HDX time periods. dA_6 exchanged rapidly during the first 10 s and then slower up to ~20 s, after which the exchange plateaued (a). dT_6 exchanged at a slower rate but kept exchanging over the entire reaction period (b).

HDX of DNA. Upon HDX, oligonucleotide m/z values ($z = 2$) shift to higher values by 0.5 for each deuterium incorporated. Figure 1 displays FT-ICR mass spectra of dA_6 (left) and dT_6 (right) doubly deprotonated anions following different HDX times. Deuterium incorporation for dA_6 is fast during the first 10 s and then slows down up to 20 s, after which it remains close to constant for the remainder of the exchange time (Figure 1a). Similar behavior was observed for dC_6, dGCATGC, and dGCATAC (see Figure 2), but to a different extent (i.e., 75% incorporation for dA_6, 74% for dC_6, 67% for dGCATGC, and 86% for dGCATAC). dT_6 (Figure 1b) and dTGGGGT (Figure 2) displayed different behavior with continuous exchange throughout the entire reaction period (up to 1 min).

Comparisons of HDX rates for all six DNA hexamers with D_2S are shown in Figure 2. Based on DNA oligomer structure, only the 5′- and 3′-hydroxyl groups can undergo HDX via the mechanism proposed for nucleoside-5′-monophosphates.[37] However, the number of experimentally observed exchanges for hexamer DNAs is much higher than two; e.g., doubly deprotonated dA_6 exchanged ~12 hydrogens with D_2S in 1 min although it only contains 5 backbone exchangeable hydrogens. This behavior indicates that the remaining exchanges must occur at nucleobases. Furthermore, if a deprotonated phosphate is located in the middle of the oligonucleotide rather than at the ends, nucleobases may be more accessible for forming a complex with D_2S than the end hydroxyl groups. Therefore, contrary to nucleoside monophosphates whose initial HDX step was proposed not to involve the nucleobases,

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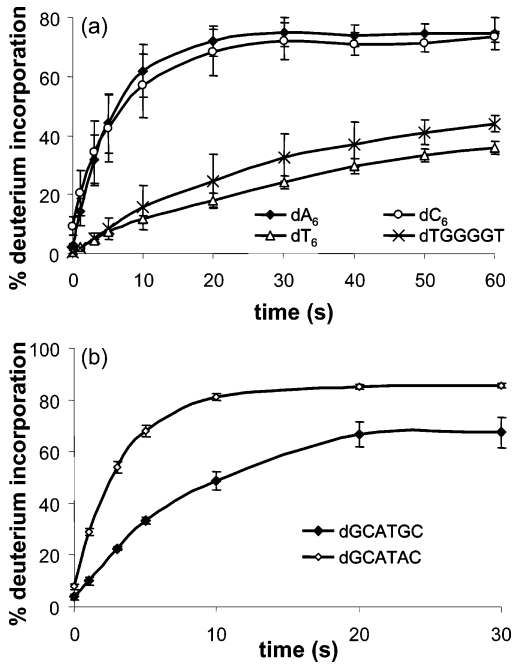
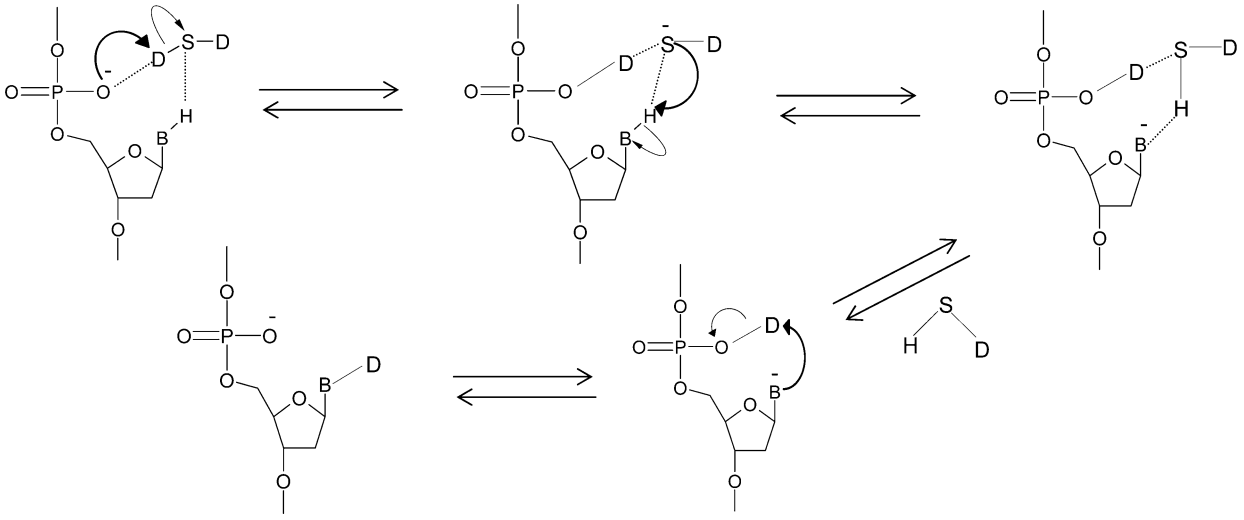


Figure 2. HDX of DNA hexamers in negative ion mode. Observed rates are $dC_6 \sim dA_6 >$ dTGGGGT > dT_6 (a) and dGCATAC > dGCATGC (b), i.e., C ~ A > G > T.

these DNA hexamers are likely having their nucelobases participate in the initial complexation step. Our experimentally observed rates were $dC_6 \sim dA_6 >$ dTGGGGT > dT_6, and dGCATAC > dGCATGC, providing the overall trend C ~ A > G > T. This behavior correlates with the expected trend, C > A > T > G, except for guanosine, which has lower gas-phase acidity than T but exchanged faster. A relay mechanism that may explain this behavior is proposed in Scheme 1. Here, D_2S forms a complex with a deprotonated backbone phosphate group and a neutral DNA nucleobase through hydrogen bonding. The phosphate group is deuterated by D_2S while the resulting DS^- anion is protonated by the nucleobase, followed by transfer of a deuteron from the phosphate group to the nucleobase. In this proposed mechanism, the faster than expected exchange observed for dG_6 may be due to the large size of guanine and the preferred orientation of its labile hydrogens (primary amine),[31,33,44] resulting in a gas-phase conformation that favors formation of an oligonucleotide-D_2S complex. On the other hand, both Freitas et al.[44] and Robinson et al.[33] found that 5′-dGMP exchanged slower than other nucleoside monophosphates, which was attributed to hydrogen bonding between guanine and the 5′-phosphate. However, several gas-phase conformations likely exist for the oligonucleotide dG_6.

HDX of RNA Compared to DNA. Because RNA has an additional hydroxyl group at the 2′ position of each sugar, it may undergo gas-phase HDX through a different mechanism than DNA. When comparing A_6 to dA_6 (Figure 3a), faster initial HDX rate was observed for the RNA ($A_6 > dA_6$). Similar behavior was seen when comparing dT_6 and U_6 (Figure 3b). This experimentally observed behavior indicates that the 2′-OH group of RNA may be involved in HDX because it constitutes the only structural difference between RNA and DNA (excluding uracil/thymine). The doubly deprotonated A_6 anion has six exchangeable hydrogens at 2′-sugar positions and five exchangeable hydrogens at backbone phosphate groups and terminal hydroxyl groups. However, it exchanged on average 13 hydrogens with D_2S within 1 min, which is more than the sum of these two groups on the backbone (11), suggesting that at least some of the exchanged hydrogens must reside on the nucleobases. HDX of RNA nucleobases may occur via the same mechanism as for DNA (Scheme 1). However, an alternative mechanism (which could occur simultaneously as the one in Scheme 1) involving the 2′-OH group is also possible. In such a mechanism, D_2S forms a complex with a deprotonated phosphate group and a 2′-OH group instead of a nucleobase, followed by intermolecular relay HDX between D_2S and the RNA backbone and intramolecular deuterium rearrangement between the phosphate group and the 2′-hydroxyl group. The faster HDX of RNA compared to DNA could be either due to higher acidity of the 2′-OH compared to that of nucleobases

Scheme 1. Proposed HDX Relay Mechanism for DNA Oligonucleotides in Negative Ion Mode[a]



[a] Exchange of nucleobases is influenced by their gas-phase acidities. The data in Figure 2 correlate well with this hypothesis, except for dG_6 (gas-phase acidity of G is lower than for T). One possible explanation is that, due to the larger size of G compared to T, a conformation favoring D_2S complexation with the phosphate and a nucleobase is more readily formed.

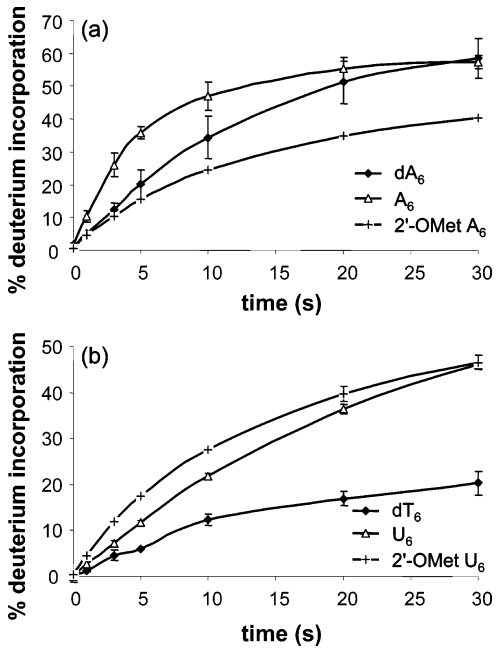
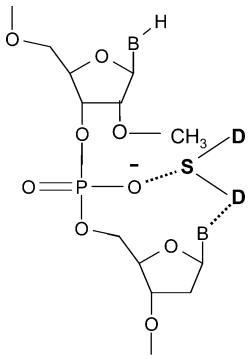


Figure 3. HDX of DNA compared to RNA and 2′-methylated RNA. Observed rates are RNA > DNA > 2′-OMet RNA except 2′-OMet U_6, which exchanges faster than dT_6.

Chart 1. Structures of 2′-OMet RNA[a]

2'-OMet RNA



[a] The methyl group at the 2′ position of 2′-OMet A_6 may induce steric hindrance, thereby impeding its complexation with D_2S, resulting in a slower HDX process compared to dA_6.

(higher acidity of the hydrogen donor group should facilitate the second step in relay HDX) or due to steric effects.

HDX of 2′-OMet RNA Compared to DNA. HDX experiments on a modified form of RNA in which the 2′-hydroxyl group is methylated (2′-OMet A_6, Chart 1) were performed to further investigate the role of the 2′-hydroxyl group in RNA HDX. Due to this methylation, there is no exchangeable hydrogen on the sugar ring, suggesting that similar exchange behavior may be observed for 2′-OMet RNA compared to DNA. However, slower exchange was observed for 2′-OMet A_6 compared to its corresponding DNA (Figure 3a). This behavior may be understood from steric hindrance by the 2′-methyl group compared to 2′-hydrogen, disfavoring formation of a complex with D_2S (Chart 1). By contrast, the modified RNA 2′-OMet U_6 displayed faster HDX than its corresponding DNA (dT_6, Figure 3b). However, the thymine methyl group may impose a similar steric effect compared to uracil, thereby counteracting the effect of the 2′ chemical group.

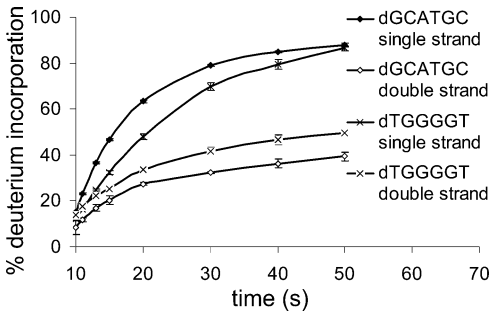


Figure 4. HDX of DNA duplexes compared to their corresponding single strands. Due to the low efficiency of duplex formation, a longer accumulation time (10 s) was used compared to the hexamers (Figures 2 and 3). Both Watson−Crick and Hoogsteen duplexes exchanged slower than their corresponding single strands because hydrogen bonds protect nucleobases from exchanging.

Compared to U_6, 2′-OMet U_6 had a very similar HDX behavior (Figure 3 b), suggesting that the Scheme 1 mechanism dominates for these oligonucleotides.

HDX of DNA Duplexes. After establishing that oligonucleotide gas-phase HDX rates depend on both nucleobase acidities, the presence of a 2′-hydroxyl group, and structural and steric effects, we set out to explore whether exchange rates can still reflect structural changes. As an example, we performed HDX of two DNA duplexes: one formed from the self-complementary oligonucleotide dGCATGC, which can form a Watson−Crick duplex, and one formed from dTGGGGT, which can form a duplex through Hoogsteen hydrogen bonding. Both these duplexes displayed slower exchange rates than their corresponding single-strand DNAs, as shown in Figure 4. Hydrogen bonding clearly protects nucleobase hydrogens from HDX, thereby revealing intriguing possibilities for characterizing higher order structure of other folded nucleic acids. Furthermore, the reduced HDX rates observed for duplexes further support our claim that nucleobases are involved in HDX of oligonucleotides.

CONCLUSION

In this work, we show that gas-phase HDX can be easily and rapidly performed in the external collision cell of a Q-FT-ICR mass spectrometer. Observed exchange behavior for DNA, RNA, and 2′-methylated RNA is consistent with relay mechanisms originating by complexation of the biomolecules with the deuterated reagent and followed by inter- and intramolecular hydrogen/deuterium transfer. We propose that negatively charged phosphate groups and neutral nucleobases both participate in the HDX process of DNA and RNA while 2′-OH groups are also involved in RNA HDX, resulting in faster exchange rates compared to DNA. The order of DNA HDX rates follows the gas-phase acidities of nucleobases, except for guanine, presumably due to its larger size and the orientation of its labile hydrogens, facilitating a gas-phase conformation that favors the formation of a complex with D_2S. Despite this complicated HDX behavior, two different kinds of DNA duplexes displayed slower exchange rates than their corresponding single strands, which also further supports the suggestion that nucleobases are involved in HDX of oligonucleotides. Thus, there is still precedent for the use of gas-phase HDX combined with mass spectrometry as a valuable technique for investigating gas-phase structures of nucleic acids. Current work in our laboratory

involves utilization of this method for determining gas-phase higher order structural stability of larger nucleic acids.

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SUPPORTING INFORMATION AVAILABLE

Representative gas-phase HDX mass spectra in addition to Figure 1 and processed raw data for Figures 2−4. This material is available free of charge via the Internet at http://pubs.acs.org.

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