2015

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Publication Details

Birrento, M. L., Bryan, T. M., Samosorn, S. & Beck, J. L. (2015). ESI-MS investigation of an equilibrium between a bimolecular quadruplex DNA and a duplex DNA/RNA hybrid. Journal of the American Society for Mass Spectrometry, 26 (7), 1165-1173.

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Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

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ESI-MS Investigation of an Equilibrium between a Bimolecular Quadruplex DNA and a Duplex DNA/RNA Hybrid

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Running Title: ESI-MS of quadruplex DNA and a DNA/RNA hybrid

Keywords: *Quadruplex DNA; quadruplex RNA; DNA/RNA hybrid; electrospray ionization mass spectrometry;, transcription; telomerase; oncogenes; transcriptional control; substrate realignment*

Abbreviations: electrospray ionization mass spectrometry, ESI-MS; NH_4OAc, ammonium acetate; ESI, electrospray ionization; qDNA, quadruplex DNA; qRNA, quadruplex RNA; circular dichroism, CD.

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Abstract

Electrospray ionization mass spectrometry (ESI-MS) conditions were optimized for simultaneous observation of a bimolecular qDNA and a Watson-Crick base-paired duplex DNA/RNA hybrid. The DNA sequence used was telomeric DNA, and the RNA contained the template for telomerase-mediated telomeric DNA synthesis. Addition of RNA to the qDNA resulted in formation of the duplex DNA/RNA hybrid. Melting profiles obtained using circular dichroism spectroscopy confirmed that the DNA/RNA hybrid exhibited greater thermal stability than the bimolecular qDNA in solution. Binding of a 13-substituted berberine (**1**) derivative to the bimolecular qDNA stabilized its structure as evidenced by an increase in its stability in the mass spectrometer, and an increase in its (CD) melting temperature of 10°C. The DNA/RNA hybrid did not bind the ligand extensively and its thermal stability was unchanged in the presence of (**1**). The qDNA-ligand complex resisted unfolding in the presence of excess RNA, limiting the formation of the DNA/RNA hybrid. Previously, it has been proposed that DNA secondary structures, such as qDNA, may be involved in the telomerase mechanism. DNA/RNA hybrid structures occur at the active site of telomerase. The results presented in the current work show that if telomeric DNA was folded into a qDNA structure, it is possible for a DNA/RNA hybrid to form as is required during template alignment. The discrimination of ligand (**1**) for binding to the bimolecular qDNA over the DNA/RNA hybrid, positions it as a useful compound for probing the role(s), if any, of antiparallel qDNA in the telomerase mechanism.

Introduction

Over the last fifteen years, the application of electrospray ionization mass spectrometry (ESI-MS) to analyse non-covalent secondary structures of DNA and its complexes with ligands has grown considerably. ESI-MS can be used to screen for interactions with drug molecules, to determine binding stoichiometries, and to compare relative stabilities of DNA-ligand complexes [1]. The first complexes analysed were double-stranded Watson-Crick base-paired DNA bound with anticancer agents such as the anthracyclines [2]. More recently, DNA quadruplexes (qDNA) have been analysed [3-5].

Quadruplex DNA (or RNA) structures are formed from the association of four guanosine residues in a stretch of contiguous guanosine residues to form a square planar arrangement via Hoogsteen base-pairing [6]. They may form from a single strand (intramolecular, monomer), or from two or four strands (intermolecular dimer or tetramer). The strands or sections of strands forming the qDNA structure may be aligned in a parallel or antiparallel orientation. The range of structures that may be formed *in vitro* or *in vivo* depends on the length of the stretch of guanosines, the nature and length of the intervening sequences and loops, and the overall length of the G-rich nucleic acid sequence with a potential to be single-stranded. Images of various types of qDNA are shown in Adrian *et al.* [6]. G-Quadruplexes may be stabilised by cations such as sodium and potassium that sit between the stacked guanosine tetrads. Importantly, for analysis by ESI-MS, ammonium ions can also function in this way.

Guanosine-rich sequences are found at telomeres and in the promoter regions of protooncogenes. Telomeres are non-coding regions of DNA at the ends of chromosomes, terminating in a single-stranded (ss) DNA overhang. With each round of DNA replication associated with cell division, the telomeres shorten because the DNA polymerase is unable to

copy DNA close to the end of the chromosome. When chromosomes reach a critical short length, this is a signal for the cell to enter senescence. In cancer cells, telomere length is maintained, either by the enzyme telomerase (~85-90% of cancers), or by another mechanism called Alternative Lengthening of Telomeres (ALT, ~10-15% of cancers) [7]. The maintenance of telomere length is necessary for immortalisation of cancer cells. For this reason, research to uncover small molecules that bind to, and either destabilize or stabilize these structures, has increased remarkably over the last 10 years [8-10] with a view to developing chemotherapeutic agents.

The first ESI-MS analyses of qDNA included relatively stable tetrameric quadruplexes formed from single strands with more than three contiguous guanosine residues [3-5]. When negatively charged Watson-Crick base-paired duplex DNA is analysed by ESI-MS, no ammonium ions remain bound to the DNA; in contrast, ESI mass spectra of more stable qDNA structures reveal the presence of bound ammonium. This is consistent with sequestration of cations in the structure and supports that some qDNA structures are sufficently stable to survive the ionization and mass analysis processes [11]. ESI-MS has been used to show that various small-molecule ligands bind to qDNA [3,12,13] and NMR spectroscopy [14] and X-ray crystallographic structures [15] support this.

RNA secondary and tertiary structures [16,17] including hybrid DNA/RNA and quadruplex RNA (qRNA), have been less extensively studied by ESI-MS. This is perhaps surprising given the important roles of RNA at all stages of expression of the genome (DNA) from DNA replication through transcription to gene silencing.

There are two RNA molecules that have particular focus in telomere biology: the intrinsic telomerase RNA (TR, hTR in humans; containing the cccuaa repeat), and telomeric repeat-containing RNA (TERRA; containing UUAGGG repeats). There have been a few studies of

TERRA by ESI-MS. Gabelica and coworkers used TERRA of different lengths (12 mer, 22 mer and 45 mer) to show that parallel RNA quadruplexes formed bimolecular assemblies (two quadruplexes) mediated by cation formation. These higher-order structures were observed at substantially higher abundance than for the analogous DNA sequences [18]. ESI mass spectra obtained by Cui *et al.* [19] of 12- and 24 mer TERRA supported the formation of inter- and intramolecular parallel quadruplexes, respectively.

There have been fewer ESI-MS studies of DNA/RNA hybrids. DNA/RNA hybrids form during the catalytic process of telomerase. The ribonucleic acid component of telomerase, TR, containing the template, associates with the complementary 3′ single-stranded overhang of telomeric DNA. This is followed by processive repeat addition synthesis of DNA in which a telomeric repeat DNA sequence is synthesised, followed by dissociation of the single-stranded product from the template, and translocation of the template (RNA) to enable synthesis of the next telomeric repeat [20-24]. The mechanism by which translocation occurs has not been fully elucidated, and the role(s), if any, of secondary DNA structures (e.g. qDNA) formed by telomeric DNA in the telomerase mechanism is not thoroughly understood [25].

The possible interaction of qDNA and RNA, relevant to telomerase activity, was investigated by Hurley and coworkers [26]. Using NMR and UV thermal denaturation analyses they showed that a thermally-induced transition of the DNA/RNA hybrid formed from telomeric DNA and hTR, d(GGTTAGGGTTAG).r(cuaacccuaacc), to a G-quadruplex DNA bimolecular structure ($d(GGTTAGGGTTAG)_2$), could occur under some conditions, supporting that qDNA formation may promote dissociation of the hybrid. This was followed up in later work where they suggested that formation of secondary structures promotes dissociation of the primer from TERT [27]. These sequences are amenable to modern ESI-

MS analysis. We have used the same hybrid and qDNA sequences and applied ESI-MS to examine the DNA/RNA hybrid ↔ qDNA equilibrium proposed previously [26]. Using the same sequences is important because it enables a direct comparison of ESI-MS with previous methods and experimental conditions, and allows an assessment of the merits of ESI-MS for studying RNA secondary structures and qDNA ↔ hybrid DNA/RNA equilibria. Under our conditions, addition of increasing amounts of complementary RNA to the bimolecular qDNA resulted in formation of hybrid DNA/RNA. In addition, we investigated the effect of binding of a novel, 13-substituted berberine derivative (**1**, shown in Supplementary Figure S1), on the relative stabilities of the hybrid and bimolecular qDNA. This compound stabilised the qDNA relative to the corresponding DNA/RNA hybrid. ESI-TWIMS was used to distinguish among ions from single-stranded d(GGTTAGGGTTAG) and ions from the bimolecular qDNA ($d(GGTTAGGGTTAG)_2$), carrying twice the number of charges, assisting in assessment of the stability of the qDNA under various conditions.

These results demonstrate the utility of ESI-MS for the investigation of equilibria among nucleic acid secondary structures including hybrid DNA/RNA and show that it is possible for RNA to compete with secondary stuctures in telomeric DNA to form an DNA/RNA hybrid as would be required for addition of the successive telomeric repeat. At the telomere, such transitions would likely be mediated through protein-nucleic acid interactions.

Experimental

Materials

MilliQ^{TM} water (Millipore, Bedford, USA) was used in all experiments. Ammonium acetate and methanol were purchased from Ajax Finechem (Seven Hills, Australia). The compound **1**

(Supplementary Figure S1) was prepared as previously described (Siritron Samosorn, Thai Petty Patent Application Number 1103000985, 16 September, 2011).

Oligonucleotides

The qDNA strand (d(GGTTAGGGTTAG), 12TAG, 3756.5 Da) was purchased from GeneWorks (South Australia) at sequencing/PCR grade. Complementary RNA (r(uaacccuaacc), 12rCU, 3698.3 Da) was purchased from Exiqon (Vedbaek, Denmark) with standard desalting. Oligonucleotides were redissolved in MilliQ^{TM} water giving a concentration of 0.5 – 1 mM prior to storage at –20 °C. The concentrations were determined by measuring the UV absorbance at 260 nm using molar absorption coefficients for 12TAG and 12rCU of 123 600 and 110 800 M^{-1} cm^{-1}, respectively.

Preparation of hybrid duplex and qDNA

The hybrid duplex was prepared by annealing equimolar amounts of the complementary RNA and DNA single strands (12rCU and 12TAG) in 0.15 M ammonium acetate (NH_4OAc), pH 6.8, to a final stock concentration of 500 μM. The solution was heated to 90 °C for 15 min and allowed to cool to room temperature. The bimolecular qDNA formed from two molecules of 12TAG, $d(GGTTAGGGTTAG)_2$, was prepared under the same conditions as the hybrid duplex using an appropriate quantity of 12TAG to form 500 μM of the bimolecular qDNA.

Preparation of ligand/nucleic acid complexes

Stock solutions of **1** (500 μM) were prepared in 60% methanol. Appropriate volumes of 0.15 M NH_4OAc, bimolecular qDNA or hybrid (DNA/RNA) duplex, and **1** were mixed to give reaction mixtures containing 10 μM or 20 μM (for ESI-MS and CD, respectively) of the qDNA or hybrid with the ligand **1** in the ratios of 1:1, 1:3, 1:6 and 1:9 (nucleic acid: ligand)

in 0.15 M NH_4OAc. The final concentration of methanol in the 1:9 mixture was 11% (vol/vol). The mixtures were equilibrated at room temperature for 10 min in a final volume of 50 μL for ESI-MS and 200 μL for CD.

Addition of RNA to Bimolecular qDNA

Single-stranded RNA (0 – 40 μM; final concentration) was mixed with 10 μM (final concentration) of qDNA in 0.15 M NH_4OAc. The reaction mixtures were equilibrated at room temperature for 10 min. When equilibrated for less than 10 min (mixing and analysis time 1 – 10 min) the results of experiments described herein were the same. To test whether RNA was able to capture ssDNA from bimolecular qDNA when it was stabilised by **1**, ligand-bound bimolecular qDNA was prepared at a ratio of 1:9 (qDNA: **1**), and equilibrated for 10 min. When equilibrated for less than 10 min (mixing and analysis time 1 – 10 min) the results of experiments described herein were the same. RNA was then added as described above.

Electrospray ionization mass spectrometry (ESI-MS) and ESI-TWIMS

Negative ion ESI mass spectra of DNA/RNA and the nucleic acid-ligand complexes were acquired using a Waters extended mass range Q-ToF Ultima^{TM} (32,000 *m/z*, Manchester, UK) mass spectrometer, fitted with a Z-spray ESI source [28]. The capillary, RF lens 1 and cone voltage were set to 2.1 kV, 65.0 V and 35.0 V, respectively. Source and desolvation temperatures of 30 and 80 °C, respectively, were used for all experiments, with a desolvation gas (N_2) flow of 300 L/hr. The pressure in the ion optics region was 3×10^{-6} mbar. Thirty-five acquisitions were combined and the resulting spectra were baseline subtracted and smoothed using the Savitzky-Golay method in the MassLynx software suite (Waters, Manchester, UK). The instrument was calibrated with 1 mg/mL cesium iodide.

Ion mobility mass spectra (TWIMS) were acquired using a Synapt G1 HDMS (Waters, Manchester, UK). The conditions were chosen to produce relative abundances of nucleic acid structures in ESI mass spectra comparable to those observed using the Q-ToF Ultima™ (above). For all experiments, the capillary, sampling cone and extraction cone voltages were set to 2.1 kV, 45.0 V and 4.0 V, respectively. The desolvation gas flow rate (N_2) was 150 mL/min with an IMS gas flow rate (Ar) of 32 mL/min. Collision energies for the trap and transfer were both set to 4.0 V and the trap bias was varied between 10 and 22 V. The wave velocity and wave height in the IMS cell were 300 m/s and 8 V, respectively. DriftScope 2.7 was used to visualise a 2D map of drift time (ms) vs *m/z* for the resulting spectra.

Circular dichroism analysis and melting experiments

Circular dichroism spectra were acquired using a Jasco J-810 spectropolarimeter fitted with a Peltier heating/cooling device. CD spectra (200 – 340 nm) were obtained using a 0.1 cm pathlength quartz cell and 20 μM of qDNA dimer or DNA/RNA hybrid in 0.15 M NH_4OAc at 25 °C. The average of 6 accumulations was recorded with a scanning speed of 100 nm/min and a data pitch of 0.1 nm. The sensitivity was set to 100 mdeg with a response time of 2 sec and a bandwidth of 1 nm. QDNA-ligand samples were prepared as described above. For acquisition of melting profiles the temperature (range of 25 °C – 85 °C) was increased at a rate of 1 °C/min. Melting was followed at 290 nm for qDNA and 260 nm for the DNA/RNA hybrid. Each cuvette was sealed with a lid and parafilm to minimize sample evaporation.

Results and Discussion

ESI-MS of Bimolecular qDNA and DNA/RNA Hybrid

One goal of this work was to determine solution and ESI-MS conditions to follow the conversion of bimolecular qDNA formed from 12TAG, $d(GGTTAGGGTTAG)_2$, to hybrid duplex DNA/RNA on addition of the complementary RNA strand. This reaction was previously studied by Salazar *et al.* using different methods and conditions [26]. ESI-MS conditions were tested separately for each nucleic acid structure; parameters that were varied included the cone voltage, desolvation gas flow rate, desolvation temperature and RF lens 1 voltage. The best conditions for observation of hybrid duplex DNA/RNA simultaneously with the qDNA are described in the Experimental section. ESI mass spectra of bimolecular qDNA formed from 12TAG, $d(GGTTAGGGTTAG)_2$, under these conditions showed peaks corresponding to $[qDNA - 5H]^{5-}$ (*m/z* 1501.5), $[qDNA + 2NH_4^+ - 7H]^{5-}$ (*m/z* 1508.4), where 'qDNA' is the bimolecular qDNA, and single-stranded (ss) DNA, $[ssDNA - 3H]^{3-}$ (*m/z* 1251.2) (Supplementary Figure S2a). The ion $[qDNA - 5\ H]^{5-}$ was the most abundant with the latter two ions present at lower, similar abundance. Circular dichroism (CD) spectra can be used to demonstrate the presence of qDNA and other nucleic acid structures in solution [29-31]. CD spectra of annealed solutions of qDNA used to obtain ESI mass spectra showed a maximum positive ellipticity at 290 nm, consistent with predominantly antiparallel qDNA [30] (Supplementary Figure S3a), confirming that qDNA was present in solution. Since ammonium ions can serve as the cation contributing to the stability of qDNA and sequestered in the G-tetrad, these ions are often preserved in the structures detected in ESI mass spectra.

The absence of ammonium ions from the bimolecular [qDNA – 5 H]$^{5-}$ (*m/z* 1501.5) suggests that they may have been lost during the ionization and/or mass analysis processes while the assembly of the two strands remained intact [11,29]. The presence of single-stranded (ss) DNA, [ssDNA – 3H]$^{3-}$ (*m/z* 1251.2) suggests that some qDNA completely dissociated. There were more gentle conditions under which [qDNA + 2NH_4^+ -7H]$^{5-}$ (*m/z* 1508.4) was more abundant than [qDNA – 5 H]$^{5-}$ (*m/z* 1501.5). For example, when the voltage of RF lens 1 was decreased from 65 to 50 V, the peak corresponding to the ammonium ion-free qDNA was very low in abundance (compare Supplementary Figure S4a and b). Accordingly, the peak at *m/z* 1251.2 decreased in abundance, consistent with diminished gas phase dissociation of the qDNA. In order to show that bimolecular qDNA could be subject to dissociation in the gas phase, a collision-induced dissociation experiment (CID) was conducted. The [qDNA – 5 H]$^{5-}$ (*m/z* 1501.5) ion was selected and subjected to increasing collision energy from 0 – 38 V (Supplementary Figure S5). At 0 V, the ion [ssDNA – 3H]$^{3-}$(*m/z* 1251.2) was negligible in abundance. When the collision energy was increased to 20 V, the abundance of [qDNA + 2NH_4^+ -7H]$^{5-}$ decreased as ammonium ions were lost from the structure. At 26 and 38 V, respectively, the ratios of the abundances of [qDNA – 5 H]$^{5-}$/[ssDNA – 3H]$^{3-}$ were approximately 2.8 and 0.3, respectively, reflecting that bimolecular qDNA dissociated to ssDNA without the dissociation of covalent bonds as the collision energy was increased. It should be noted that this ion (*m/z* 1251.2) can also be assigned as [qDNA - 6H]$^{6-}$. In all cases, the abundance of the 6- ion of bimolecular qDNA with bound ammonium ions (e.g. [qDNA + 2NH_4^+ - 8H]$^{6-}$; calculated *m/z* 1256.8) was negligible, suggesting that this ion does not readily form/is relatively unstable, and supporting that, under the ESI-MS conditions applied in these experiments, the ion at *m/z* 1251.2 arises predominantly from [ssDNA – 3H]$^{3-}$ rather than [qDNA - 6H]$^{6-}$. Ion mobility mass spectra (ESI-TWIMS) of the qDNA were acquired using a Synapt (G1) HDMS under a range of conditions. When the bias was set to 10 V,

peaks at *m/z* 1251.1 corresponded to ssDNA and bimolecular qDNA (Supplementary Figure S6a), showing that under some gas phase conditions using this instrument it was possible to observe $[qDNA - 6H]^{6-}$. When the bias was increased to 22 V, the peak at *m/z* 1251.1 corresponded only to ssDNA.

In all conditions tested, only one peak of significant abundance was observed in ESI mass spectra (Supplementary Figure S2b) of the DNA/RNA hybrid: $[DNA/RNA – 5H]^{5-}$ (*m/z* 1489.9). As expected, ammonium ions were not detected in the structure as this hybrid is a Watson-Crick base-paired duplex stabilised by H-bonding among complementary nucleobases (u.A and C.G). The CD spectrum (Supplementary Figure S3a) was in agreement with that previously obtained using a similar hybrid sequence [32]. Since the qDNA structure with two bound ammonium ions was more abundant in the ESI mass spectra acquired when the RF lens 1 voltage was lower (50 V), these conditions were applied to determine if they were also suitable for analysis of the hybrid DNA/RNA duplex. Under this condition (other parameters remaining the same), the ion at *m/z* 1489.9 was present at very low abundance (compare Supplementary Figure S4c and d), making it unsuitable for observation of qDNA and the hybrid in the same mass spectrum. Therefore, the conditions described in the Experimental Section represent a compromise (RF lens 1, 65 V) that enabled analysis of qDNA (with and without bound ammonium ions) and hybrid DNA/RNA in the same ESI mass spectrum.

Addition of RNA to Bimolecular qDNA

To compare the relative stabilities of the DNA/RNA hybrid and bimolecular qDNA, RNA (12rCU) was added in increasing amounts to pre-formed qDNA. Figure 1 shows the ESI mass spectrum of the bimolecular DNA alone (a), and with increasing amounts of added RNA (b and c). In the 1:1 mixture (qDNA: DNA/RNA hybrid, Figure 1c), the peak

corresponding to the hybrid is approximately three times that of the bimolecular qDNA. As RNA was added to the qDNA, the relative abundance of the ions from bimolecular qDNA, $[qDNA - 5\ H]^{5-}$ (*m/z* 1501.5), and $[qDNA + 2NH_4^+ - 7H]^{5-}$ (*m/z* 1508.4) decreased, supporting the loss of the qDNA structure in solution. When RNA was added in molar excess over the bimolecular qDNA, ions corresponding to qDNA were negligible in abundance and a peak from free ssRNA was observed (*m/z* 1231.1, not shown). The results were expressed graphically by summing the abundances of the qDNA ions (with and without bound ammonium ions) and expressing them as a percentage of the abundance of all 5- ions in the spectrum (qDNA + DNA/RNA) and comparing these with the relative abundance of the ion from the DNA/RNA hybrid (Figure 1d). It should be noted that while this Figure shows the decrease in the qDNA corresponding with an increase in the DNA/RNA hybrid with the addition of RNA as observed in the ESI mass spectra, the response factors (ionization efficiency coupled with efficiency of transfer of ions through stages of mass analysis and detection) of the DNA/RNA hybrid and qDNA may differ. If this is the case, then these data are not amenable to quantitative analyses. Nevertheless, these results show that RNA was able to base pair with DNA of the bimolecular qDNA and form a DNA/RNA hybrid. When the hybrid was preformed and treated with increasing amounts of bimolecular qDNA, the analogous results were obtained with the ESI mass spectrum of the 1:1 mixture indistinguishable from that shown in Figure 1c. Furthermore, when these mixtures were left to equilibrate for one day or four days at room temperature, the ESI mass spectra were unchanged (not shown; these time periods were chosen for comparison with the results of Salazar *et al*., [26] described below).

Relative stabilities of Bimolecular qDNA and DNA/RNA hybrid

Melting profiles obtained by CD spectroscopy supported that the thermal stability of the DNA/RNA hybrid in 0.15 M NH_4OAc was greater than that for the bimolecular qDNA under the conditions of our experiments (Supplementary Figure S3b). The temperatures at which half of the qDNA or hybrid DNA/RNA duplex was denatured, the melting temperatures, were ~37 °C and 57 °C, respectively.

Previously, Salazar *et al.* [26] examined the same oligonucleotide sequences as in the present work as a model for the hybrid that forms to enable extension of the telomere primer on the telomerase RNA template. At high oligonucleotide concentrations (5 mM), NMR spectra acquired in the presence of 100 mM K^+ showed that the hybrid denatured as it was heated to 50 °C, where a new set of peaks were observed that were proposed to correspond to parallel G-quadruplex DNA. In Na^+, the hybrid was present in greater abundance, but denatured as the sample was heated to 75 °C with no evidence for the formation of a G-quadruplex at any temperature. These researchers proposed that under their conditions in K^+-containing buffer, and after the thermally-induced transition from hybrid to G-quadruplex, an equilibrium was established between the two secondary structures; after 4 days, the amount of G-quadruplex diminished with a concomitant increase in the amount of the hybrid. The melting temperatures and relative stabilities of the hybrid and G-quadruplex DNA were shown to be affected by oligonucleotide and cation concentrations. The strand orientations (parallel or antiparallel) of the qDNA formed under these different conditions were not assessed by NMR.

The current work also supports that an equilibrium can be established between the hybrid and qDNA, but in contrast with the results of Salazar *et al*. [26], the hybrid was substantially more thermally stable than the qDNA, and the qDNA strands were predominantly

antiparallel. Salazar *et al*. proposed a four-stranded parallel qDNA, although that work [26] was carried out before it was possible to use ESI-MS to determine strand stoichiometry. Their proposal is reasonable given the high DNA concentration (5 mM) and different cations used in their work. Such differences in annealing conditions are known to result in qDNA with different strand stoichiometry and orientation (parallel *cf.* antiparallel). In a proposed mechanism for the activity of telomerase [33], an RNA hybrid must be formed at two steps: (1) hTR aligns with the 3′ end of the telomeric primer DNA forming a duplex hybrid; the 5′ end of the telomeric DNA is proposed to interact with a domain of the TERT (telomerase) protein, and (2) after nucleotide addition, the DNA/RNA hybrid undergoes strand separation (outside the active site), and the telomeric DNA must re-anneal to the alignment region of the RNA to allow repeat addition of nucleotides; the realigned DNA/RNA hybrid is then recaptured by the active site. The results presented in the current work show that if telomeric DNA was folded into a qDNA structure, that it is possible for a DNA/RNA hybrid to form (e.g. at step (1)). Based on their results, Hurley and co-workers [26] suggested that formation of qDNA might promote dissociation of the hybrid (e.g. at (2)). Together these studies and the current work provide a basis for understanding that whether telomerase action is promoted or inhibited will depend on the relative stabilities of the DNA/RNA hybrid compared with any qDNA that forms under the prevailing conditions. Since the forward and reverse processes of the equilibrium between the hybrid and the qDNA have been demonstrated under different conditions *in vitro*, the results of the two studies (current and Salazar *et al.* [26]) taken together suggest, as expected, that the identity of the predominating nucleic acid secondary structure will depend on the microenvironment; for example, the presence and nature of protein-nucleic acid interactions at the active site of telomerase or outside it, as proposed during translocation.

Effect of ligand (1) on stabilities of Bimolecular qDNA and DNA/RNA hybrid

Previously, 13-substituted berberine derivatives were shown to stabilise various qDNA structures [4,13]. Some qDNA-binding ligands inhibit telomerase activity/processivity, and have been proposed as leads for potential anticancer chemotherapeutics [34]. A new compound (**1**), (Supplementary Figure S1) was tested for its ability to bind to the bimolecular qDNA and the hybrid. Figures 2a and c show the ESI mass spectra (*m/z* 1450 – 1750) of qDNA and the hybrid alone, respectively, for comparison with the spectra below (b and d) where a 3-fold molar excess of ligand **1** was added to the solutions. In the 1:3 qDNA: ligand mixture (Figure 2b), the most abundant ion (*m/z* 1600.0) is from the complex where there are two ammonium ions present and one molecule of ligand bound: $[qDNA + 2NH_4^+ + (\mathbf{1}) - 7H]^{5-}$. There is also an ion of low to moderate abundance from $[qDNA + 2NH_4^+ + 2(\mathbf{1}) - 7H]^{5-}$ (*m/z* 1692.4). The increase in abundance in the ESI mass spectra of qDNA with bound ammonium ions when ligand was present compared with when it was absent (*cf.* Figures 2b and a, respectively), suggests that the ligand (**1**) stabilised the ammonium ion-bound form of the bimolecular qDNA, preventing the ammonium ions from dissociating in the mass spectrometer. This is supported by closer inspection of the relative abundances of qDNA + n(**1**), $qDNA + NH_4^+ + n(\mathbf{1})$ and $qDNA + 2NH_4^+ + n(\mathbf{1})$, where n = 0 – 2, as a function of increasing ligand concentration (Supplementary Figure S7). As ligand concentration increased, the amount of qDNA with two bound ammonium ions detected in the mass spectra increased while the qDNA with no bound ammonium ions decreased, consistent with the proposal that ligand (**1**) stabilizes $qDNA + 2NH_4^+$.

Figures 2c and d show the ESI mass spectra of the DNA/RNA hybrid alone and with a 3-fold molar excess of ligand **1**, respectively. In contrast with the bimolecular qDNA, the most abundant ion was from free nucleic acid (hybrid). There was also an ion of low to moderate

abundance corresponding to the DNA/RNA hybrid with one ligand molecule bound (*m/z* 1581.7, [DNA/RNA + (**1**) – 5H]$^{5-}$). The relative abundances of all complexes detected for qDNA and the hybrid, respectively, as judged by the ESI mass spectra are shown in Supplementary Figure S8. The ligand bound more extensively to the qDNA than the hybrid, with the complex with two ligands bound (qDNA + 2(**1**)), the most abundant in the 1:9 mixture. The DNA/RNA hybrid bound the ligand relatively weakly with substantial amounts of free hybrid duplex (~50% of all complexes) present in the 1:9 mixture; the complex where one ligand molecule was bound was the most abundant. These observations are consistent with binding of the ligand stabilizing the qDNA against dissociation, and preferential binding of the ligand to the bimolecular qDNA over the duplex DNA/RNA hybrid.

The difference in melting temperatures of the qDNA (37 °C) and DNA/RNA hybrid (57 °C) was substantial. The effects of ligand (**1**)-binding on the thermal stability of the qDNA and its resistance to unfolding and hybridization with RNA were tested. Ligand (**1**) was added to qDNA to give qDNA: ligand (**1**) mixtures (1:1, 1:3, 1:6 and 1:9). As the concentration of the ligand was raised, the melting temperature (thermal stability) of the qDNA, as judged by CD spectroscopy, increased. The melting temperature increased from 37 °C (no ligand) to 47 °C in the 1:9 mixture (Figure 3). The experiment presented in Figure 1 was repeated, but using bimolecular qDNA that was treated with a 9-fold molar excess of the ligand (**1**). Under these conditions the most abundant complex is expected to contain at least two ligands bound to qDNA (qDNA + 2NH_4^+ + 2(**1**); see Supplementary Figure S8). Figure 4a-c shows ESI mass spectra of qDNA-ligand (**1**) mixtures with increasing amounts of RNA added (up to equimolar amounts of RNA and qDNA). Figure 4d shows a graph comparable to that shown in Figure 1d (when no ligand was present). When ligand (**1**) was bound to the qDNA, qDNA was more abundant than the DNA/RNA hybrid at all RNA concentrations (Figure 4d). Comparison of these two graphs (Figures 1d and 4d) shows that the binding of the ligand

stabilized the qDNA against unfolding and therefore inhibited hybridization of dissociated DNA with RNA. The stabilization of qDNA against unfolding and hybridization with complementary DNA in the presence of qDNA-binding ligands has been observed in a range of studies; for example by de Pauw and co-workers [35].

Closer inspection of the abundances of individual ions (free RNA $[RNA - 3H]^{3-}$, *m/z* 1231.7; qDNA ($[qDNA - 5\ H]^{5-}$, *m/z* 1501.5, and $[qDNA + 2NH_4^+ - 7H]^{5-}$, *m/z* 1508.4; ssDNA $[ssDNA - 3H]^{3-}$, *m/z* 1251.2; DNA/RNA hybrid duplex ($[DNA/RNA - 5H]^{5-}$, *m/z* 1489.9) as increasing amounts of RNA were added to either qDNA, or qDNA in the presence of the ligand, revealed more detailed information about the stability of the qDNA under the different conditions (not shown). When the ligand was present, the abundance of ssDNA was low supporting that the ligand stabilized the qDNA against dissociation in the mass spectrometer. In the presence and absence of the ligand, the abundance of ssDNA (*m/z* 1251.2) decreased as RNA was increased, supporting that DNA that is H-bonded in the hybrid is more stable to the mass spectrometry conditions than the qDNA. In agreement with this, the abundance of free RNA (*m/z* 1231.7) was greater when the ligand was present.

These results, together with the observation that the ligand does not bind extensively to the hybrid, suggests that **1** (Supplementary Figure S1) will be a useful compound for probing the role(s), if any, of antiparallel qDNA in the telomerase mechanism.

Conclusions

A DNA/RNA hybrid duplex representing the template RNA of telomerase in alignment with telomeric DNA, was readily detected and analysed by ESI-MS. The optimised ESI-MS conditions for analysis of the bimolecular qDNA ($d(GGTTAGGGTTAG)_2$), used in this work, were not suitable for ESI-MS observation of the duplex DNA/RNA hybrid. In order to observe conversion of the qDNA to the hybrid in the same mass spectrum, a compromise was

made in ESI-MS conditions. ESI-MS was used to show that bimolecular qDNA can unfold to form the hybrid, demonstrating that if qDNA forms during catalysis by telomerase that it is possible for realignment with the RNA template to occur. The binding of a 13-substituted berberine derivative (**1**) to the bimolecular qDNA stabilized the structure against thermal melting and unfolding in the presence of RNA to form the hybrid. Ligand (**1**) did not bind to the DNA/RNA hybrid. The ability of ligand (**1**) to discriminate among qDNA and hybrid means this compound might be used to probe mechanism of telomerase. In an experimental program that is currently under way, this and other compounds that selectively bind different DNA secondary structures (e.g. parallel or antiparallel qDNA) are being used to probe their effect if any, on telomerase activity and/or processivity.

Acknowledgements

M.L.B. acknowledges receipt of an Australian Postgraduate Award. The mass spectrometer and CD spectropolarimeter used in this work were funded by the Australian Research Council (LE0453832 and LE0560751, respectively). S.S. acknowledges Srinakharinwirot University Research Grant Number 062/2554. The work was also supported by Cancer Council NSW (project grant RG 11-07 to T. B.) and Cancer Institute NSW (Fellowship 11/CDF/3-05 to T. B.).

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Figure Legends

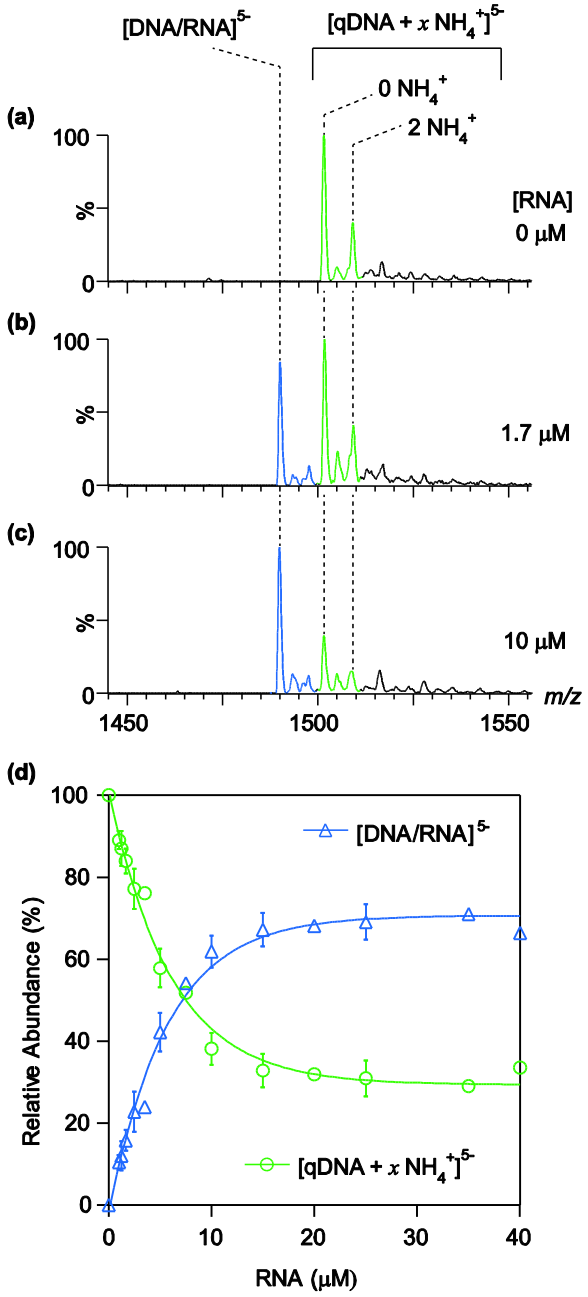


Figure 1: ESI mass spectra showing addition of RNA (0 – 10 µM) to bimolecular qDNA (10 µM) to form a DNA/RNA hybrid. (a) qDNA alone, (b) 6:1 mixture of qDNA: RNA (12rCU) (c) 1:1 mixture of qDNA: RNA (12rCU), and (d) relative abundances (5- ions) of bimolecular qDNA (green) and hybrid DNA/RNA (blue) as judged by ESI mass spectra. Average of 3 experiments; error bars represent ± 1 standard deviation. In the ESI mass spectra qDNA peaks are in green and hybrid DNA/RNA peaks are blue; *x* is the number of ammonium ions in the structure.

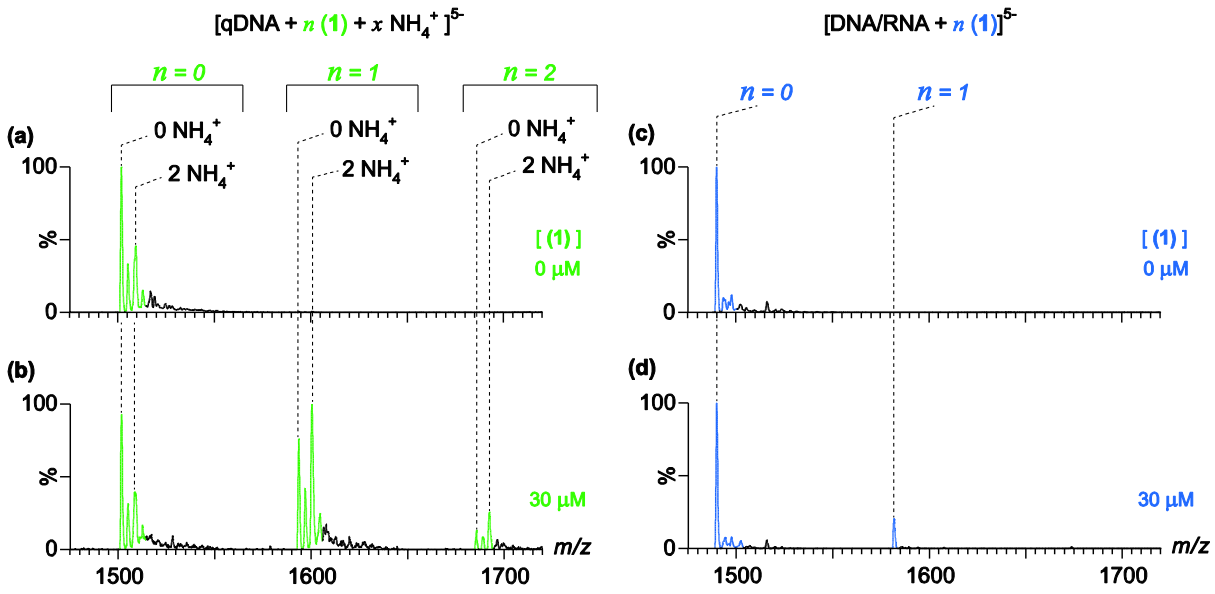


Figure 2: Addition of 13-substituted berberine derivative (**1**) to bimolecular qDNA (10 µM) or DNA/RNA hybrid (10 µM). (a) qDNA alone, (b) 1:3 mixture of qDNA: **1**, (c) hybrid DNA/RNA alone, (d) 1:3 mixture of hybrid DNA/RNA: **1**; *x* is the number of ammonium ions in the structure; *n* is the number of ligand (**1**) molecules bound.

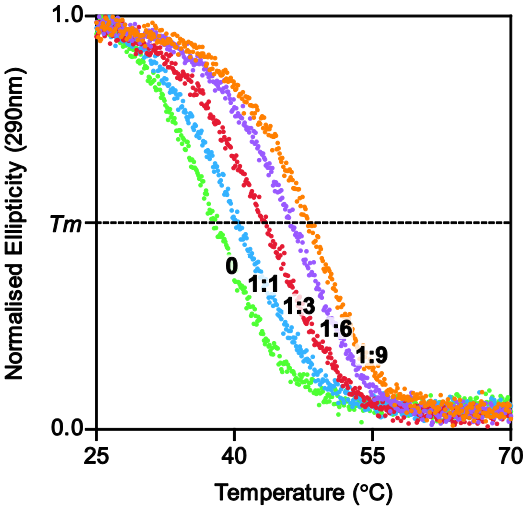


Figure 3: CD melting curves of qDNA alone and 1:1, 1:3, 1:6 and 1:9 mixtures with ligand (**1**).

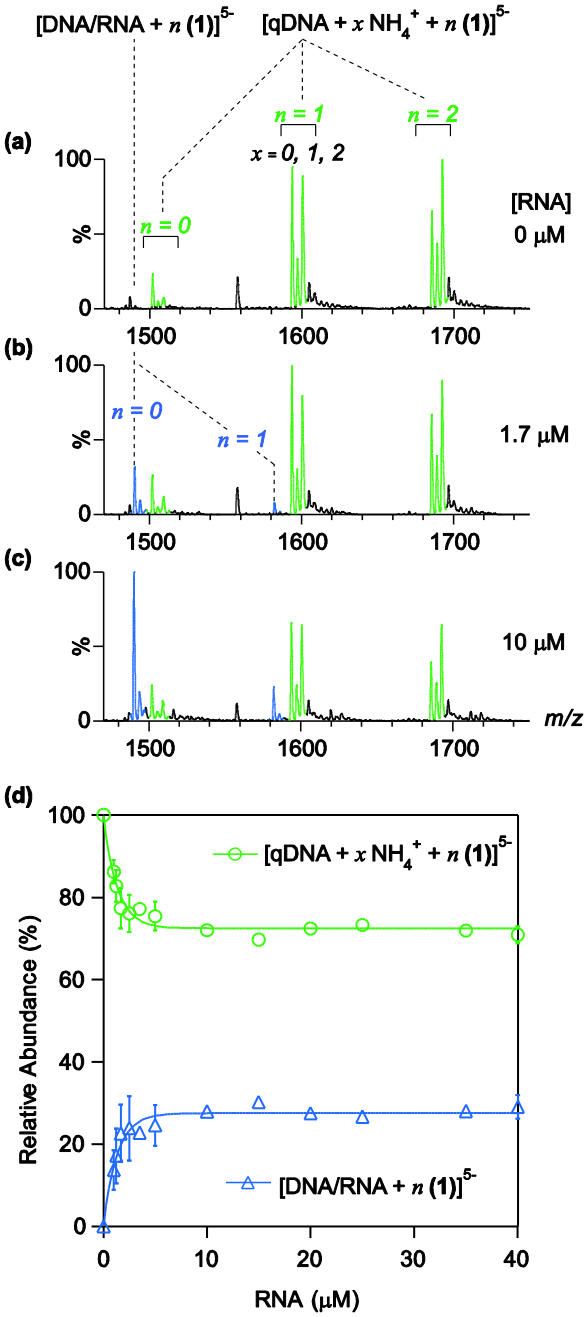
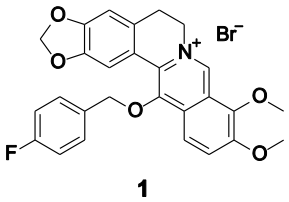


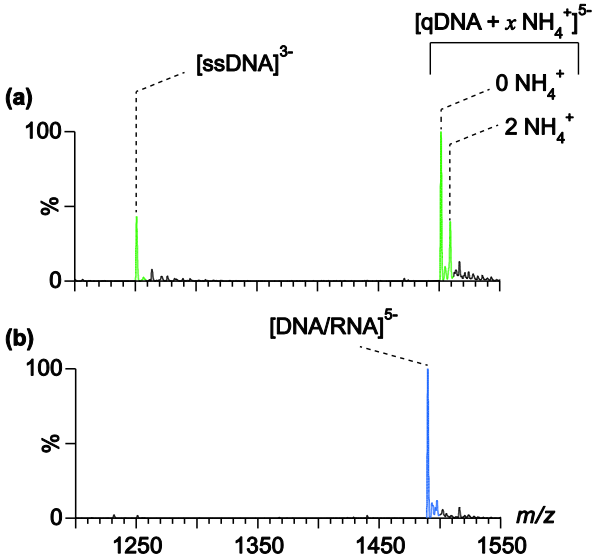
Figure 4: ESI mass spectra showing addition of RNA to bimolecular qDNA to form an DNA/RNA hybrid in the presence of a 9-fold excess of ligand (**1**). (a) qDNA alone, (b) 6:1 mixture of qDNA: RNA (12rCU) (c) 1:1 mixture of qDNA: RNA (12rCU), and (d) relative abundances (5- ions) of bimolecular qDNA (green) and hybrid DNA/RNA (blue) as judged by ESI mass spectra. Average of 3 experiments; error bars represent ± 1 standard deviation. In the ESI mass spectra qDNA peaks are in green and hybrid DNA/RNA peaks are blue; *x* is the number of ammonium ions in the structure; *n* is the number of ligand (**1**) molecules bound.

Supplementary Figures

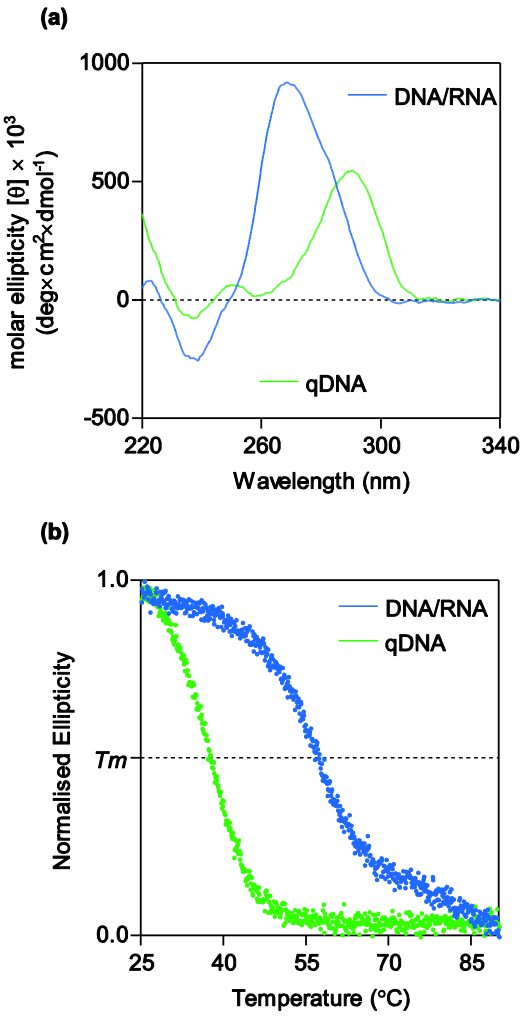


1

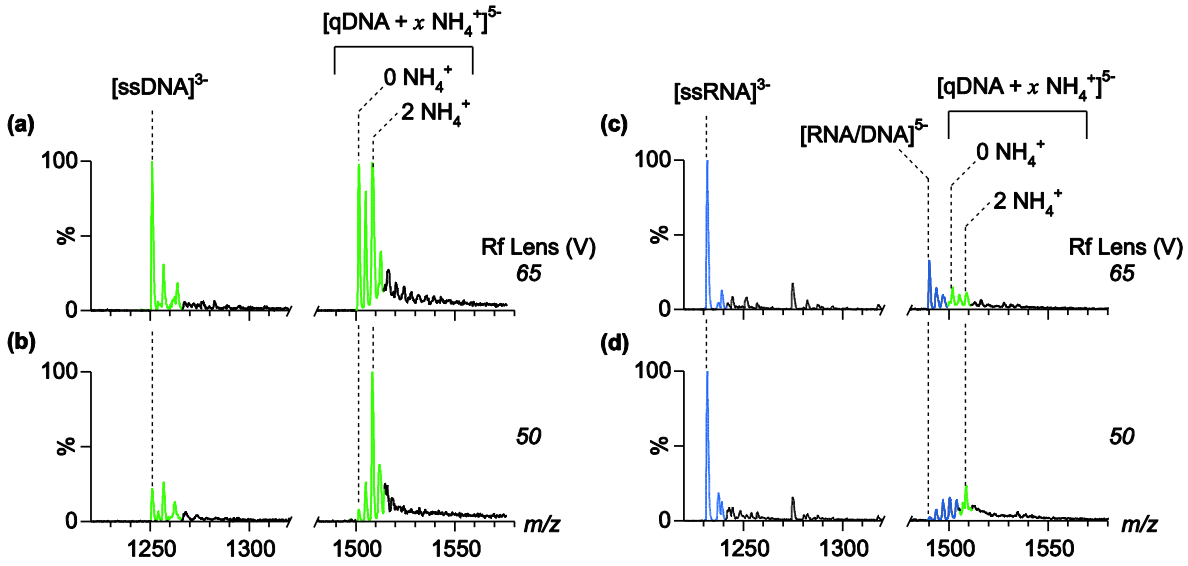
Supplementary Figure S1: Structure of **1**, a 13-substituted berberine derivative used in this work.



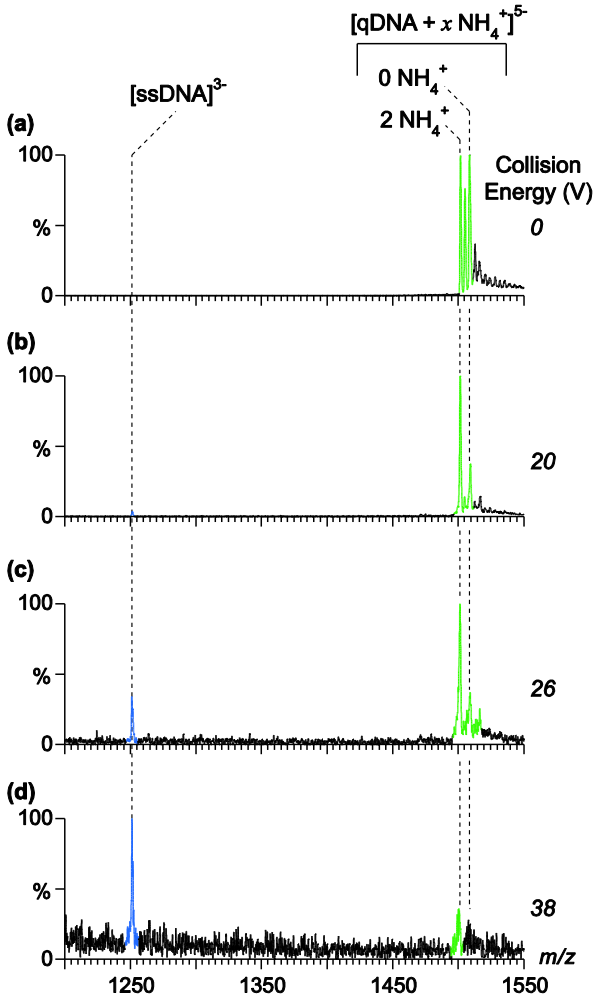
Supplementary Figure S2: ESI mass spectra of: (a) bimolecular qDNA d(GGTTAGGGTTAG)$_2$ (green) and (b) DNA/RNA hybrid, d(GGTTAGGGTTAG).r(cuaacccuaacc) (blue). Ions observed were [qDNA – 5 H]$^{5-}$ (*m/z* 1501.5), [qDNA + 2NH$_4^+$ -7H]$^{5-}$ (*m/z* 1508.4), [ssDNA – 3H]$^{3-}$ (*m/z* 1251.2), [DNA/RNA – 5H]$^{5-}$ (*m/z* 1489.9); *x* refers to the number of ammonium ions.



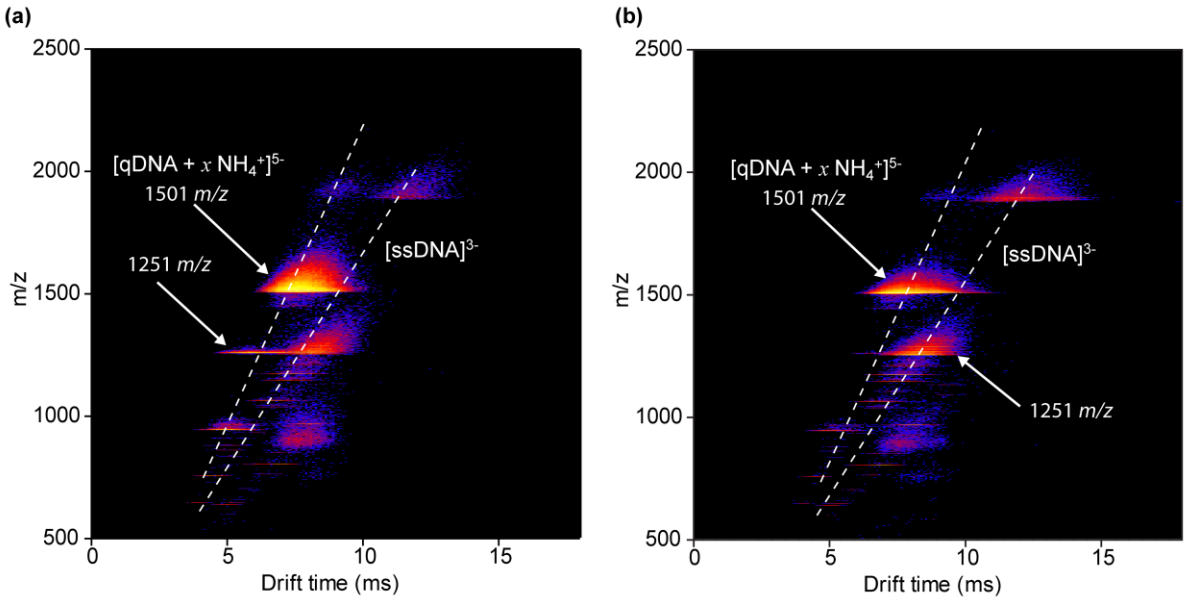
Supplementary Figure S3: (a) Circular dichroism (CD) spectra of the DNA/RNA hybrid, d(GGTTAGGGTTAG).r(cuaacccuaacc), (blue) and dimeric qDNA d(GGTTAGGGTTAG)$_2$ (green) at 10 and 20 μM (molecular concentration), respectively, in 0.15 M NH_4OAc. (b) CD melting profiles for the DNA/RNA hybrid, d(GGTTAGGGTTAG).r(cuaacccuaacc), (blue, 260 nm) and qDNA d(GGTTAGGGTTAG)$_2$ (green, 290 nm).



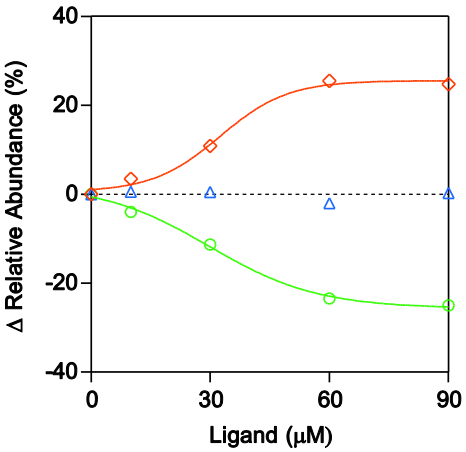
Supplementary Figure S4: ESI mass spectra showing the effect of changing RF Lens voltage on qDNA and DNA/RNA hybrid. (a) qDNA (green) with RF Lens 65 V and (b) RF lens 50 V. (c) qDNA (green) with addition of excess (15 μM) ssRNA (blue) added to form hybrid and analysed using at RF lens 65 V and (d) RF Lens 50 V; *x* refers to the number of ammonium ions.



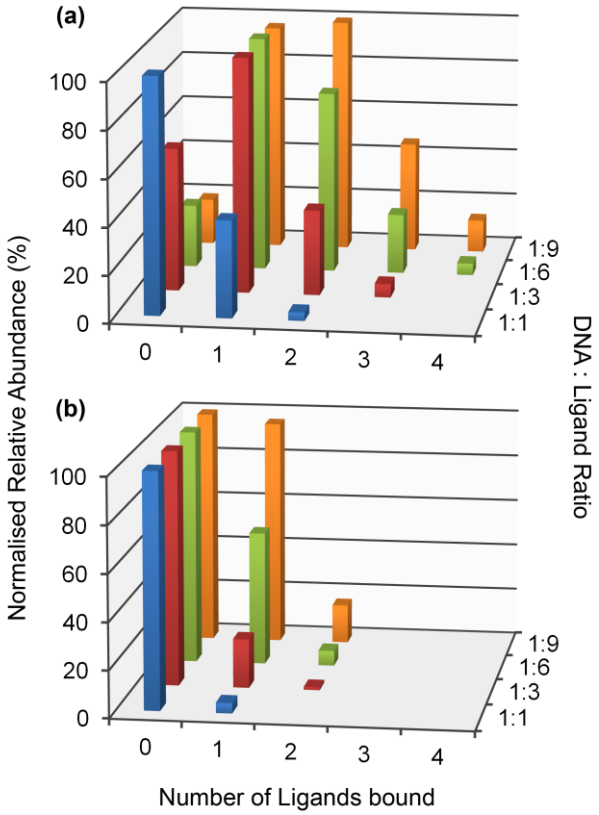
Supplementary Figure S5: ESI-MS/MS (Q-ToF *Ultima*) showing the dissociation of bimolecular qDNA (5- ions; green) to ssDNA (3- ions; blue) with increasing collision energy voltage. (a) 0 V (b) 20 V (c) 26 V (d) 38 V. Since the instrument had an extended mass range (*m/z* 32,000), it was not possible to select only *m/z* 1501.5 as the precursor ion (loss of resolution in quadrupole with decrease in frequency); all 5- ions were transmitted: $[qDNA - 5\ H]^{5-}$, $[qDNA + 1NH_4^+ - 6H]^{5-}$ and $[qDNA + 2NH_4^+ - 7H]^{5-}$. As the collision energy was increased, ammonium ions were lost from $[qDNA + 1NH_4^+ - 6H]^{5-}$ and $[qDNA + 2NH_4^+ - 7H]^{5-}$; at the highest collision energies, $[qDNA - 5\ H]^{5-}$ dissociated to $[ssDNA - 3H]^{3-}$ (*m/z* 1251.2).



Supplementary Figure S6: Negative ESI-TWIMS Driftscope images of bimolecular qDNA d(GGTTAGGGTTAG)$_2$ using the conditions described in the Experimental section. (a) Bias = 10 V; (b) Bias = 22 V. Dotted white lines show charge states corresponding to each nucleic acid structure: bimolecular qDNA (qDNA + xNH$_4^+$), and ssDNA; x refers to the number of ammonium ions.



Supplementary Figure S7: Change in relative abundances of qDNA + n(1) (green circles), qDNA + NH_4^+ + n(1) (blue triangles) and qDNA + $2NH_4^+$ + n(1) (orange diamonds) as a function of increasing ligand (1) concentration (n = 0, 1). The change in relative abundance is calculated by subtracting the abundance of ions from qDNA with no ligand bound from the abundances of all ions from qDNA (with and without ligand bound). At all concentrations of ligand, ions from qDNA with no ammonium ions decrease in abundance and ions from qDNA with two bound ammonium ions increase in abundance consistent with the proposal that ligand (**1**) stabilizes qDNA + $2NH_4^+$.



Supplementary Figure S8: Normalised relative abundances of qDNA-ligand (**1**) and DNA/RNA-ligand (**1**) complexes in 1:1, 1:3, 1:6 and 1:9 mixtures. (a) qDNA-ligand (**1**) complexes, and (b) hybrid DNA/RNA-ligand (**1**) complexes. The abundances of all ions from qDNA-ligand complexes (a) or DNA/RNA hybrid-ligand complexes (b) were summed and expressed as a percentage of the sum of the abundances of all the ions in the spectrum excluding the ion at 1251.2 from ssDNA if present, in mixtures containing qDNA; normalised to abundance of base peak in each spectrum.