SHORT COMMUNICATION

Positive Ion Electrospray Ionization Mass Spectrometry of Oligonucleotides

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Positive ion electrospray ionization mass spectra have been obtained of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA), including transfer RNAs (77-mer, ~ 25 kDa). For several different solution conditions, the charge state distributions of DNA and RNA molecules were determined. It is postulated that the production of the multiply charged positive ions results from gas phase dissociation of complexes between nitrogen-containing bases and oligonucleotides. © *1997 American Society for Mass Spectrometry (J Am Soc Mass Spectrom 1997, 8, 90–95)*

Electrospray ionization mass spectrometry (ESI-MS) is a powerful technique for analyzing biological molecules [1–3]. Although the electrospray ion formation process is not completely understood [4], the ions detected in the gas phase are thought to reflect the ions present in solution [5, 6]. However, it is possible that gas phase reactions can contribute to the ion formation process of electrospray ionization.

Because proteins contain acidic and basic amino acid residues, the solution conditions generally determine whether positive or negative ions will be observed. Typically, for optimum sensitivity in protein analysis, basic solution conditions are used for negative ion ESI-MS [7] and acidic solutions are used for positive ion ESI-MS. However, proteins in high pH solutions (basic) have been observed to produce multiply charged positive ions [8–10].

For deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) molecules, the purine and pyrimidine bases can be protonated, deprotonated or neutral depending on the solution conditions. However, the phosphate groups ($pK_a < 1$) on the sugar backbone will be deprotonated under all solution conditions, leading to a net negative charge on DNA and RNA molecules. Thus, DNA and RNA molecules are typically studied using electrospray ionization mass spectrometry in the negative ion mode [11–16]. Nucleotides (e.g., AMP and ADP [17]) and small DNA molecules [e.g., $d(pA)_3$, unpublished results] can carry 1 to 2 positive charges and have been observed by positive ion ESI-MS. Recently, we have been able to observe positive ions of relatively large (up to 25 kDa) DNA and RNA molecules using ESI-MS. Understanding how positive ions of DNA and RNA are observed may shed some light on the electrospray ion formation process.

Being able to study DNA and RNA in the positive ion mode with electrospray ionization may have some practical advantages. For example, the instrument can be tuned once for positive ions for both protein and DNA/RNA analysis. The direct observation of protein binding to DNA/RNA and determination of binding stoichiometry may be an important application of ESI-MS [18–20]. Positive ion ESI-MS analysis could provide an alternative mode for observing all possible products for protein-DNA/RNA noncovalent binding reactions (i.e., free protein, free oligonucleotide, and protein-DNA/RNA complex). It is also possible that DNA and RNA sequencing by collisionally activated dissociation (CAD) [12, 13] may be more efficient for multiply charged positive ions than negative ions.

The charge state distributions of several DNA and RNA molecules were studied using ESI-MS in the positive ion mode. The pH range of the solutions studied was 3–10. An explanation for observation of positive ions of DNA and RNA consistent with experimental data is presented.

Experimental

Instrumentation

ESI mass spectra were acquired with a double focusing mass spectrometer with a mass-to-charge (m/z) range of 10,000 (at 5 kV full acceleration potential) [21]. The Finnigan MAT 900Q forward geometry hybrid mass

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spectrometer (Bremen, Germany) is equipped with a position-and-time-resolved-ion-counting (PATRIC) scanning focal plane detector. A heated glass capillary ESI inlet was used. A countercurrent stream of warm nitrogen gas (~ 60 °C) and gas phase collisions in the ESI interface, controlled by adjustment of the voltage difference between the tube lens at the exit of the glass capillary and the first skimmer element (ΔV_{TS}), helped desolvate the ESI-produced droplets. A stream of SF_6 coaxial to the spray suppressed corona discharges.

Chemicals

The ammonium salts of $d(pA)_9$, $d(pT)_9$, $d(pT)_{12}$, $d(pT)_{18}$, $d(pC)_9$, and $d(pC)_{18}$ and the sodium salts of $d(pT)_3$ and $d(pT)_6$ were purchased from Sigma Chemical (St. Louis, MO, USA). These oligonucleotides were used without further purification. The transfer RNAs, $tRNA^{fmet}$ (*E. Coli*) and $tRNA^{phenylalanine}$ (brewer's yeast), were purchased from Sigma and purified by ethanol precipitation as an ammonium salt [11, 22]. Ψ-RNA stem-loop 1, Ψ-RNA stem-loop 2, TAR 31-mer, and TAR 28-mer were chemically synthesized [23], and purified by gel electrophoresis and ethanol precipitated as ammonium salts. The concentration of RNA was determined by UV spectrophotometry.

Sample Preparation

For the homo-oligonucleotides, 0.1 mM stock solutions were prepared in 10 mM ammonium acetate (NH_4OAc, pH 6). ESI-MS solutions were prepared at a concentration of 40 pmol/μL in 10% (*v/v*) methanol. The pH was adjusted to pH 3, 6, or 10 by addition of acetic acid, 10 mM NH_4OAc, or ammonium hydroxide, respectively. In addition, ESI-MS solutions were prepared in 10 mM aniline, imidazole, diethylamine, and triethylamine. For TAR 28-mer, TAR 31-mer, EcoR1, $tRNA^{fmet}$ and $tRNA^{phenylalanine}$, ESI-MS solutions (10 pmol/μL) were prepared in a similar manner. For Ψ-RNA stem-loop 1 and stem-loop 2, a 13 pmol/μL stock solution was prepared in 10 mM NH_4OAc, 10% (*v/v*) methanol and 0.25 mM 1,2-cyclohexanediamine tetra-acetic acid (CDTA) [22]. The pH 10 solution was prepared by adding 20 μl of 2.5% NH_4OH and 20 μL acetonitrile to 60 μL of the stock solution. For the pH 6 solution, the stock solution was used. The pH 3 solution was prepared by adding 40 μL of 80/15/5 acetonitrile/water/acetic acid (*v/v/v*) to 60 μL of the stock solution.

Results and Discussion

Several ribonucleic acids were available to us for mass spectrometric analysis. We have been using ESI-mass spectrometry to study protein-RNA recognition events [19]. Tat protein from human immunodeficiency virus (HIV) is a viral trans-activator that is essential for viral replication. Tat is required to increase the rate of transcription from the HIV long terminal repeat (LTR) and its action is dependent on the region near the start of transcription in the viral LTR called the trans-activation responsive (TAR) element. TAR RNA contains a three nucleotide bulge that is essential for Tat binding and activity. HIV-1 nucleocapsid protein (NCp7) is responsible for encapsulation and packaging of genomic Ψ-RNA during viral particle assembly. A 44-mer RNA containing the HIV-1 Ψ region is folded into two stem-loop structures. TAR RNA and RNAs corresponding to the individual step-loop structures of Ψ-RNA were synthesized for these studies. The structures are shown in Figure 1. In Table 1, the maximum and average positive charge observed by ESI-MS are listed for the different solutions conditions studied. Above pH 6, the solution pH did not affect the maximum or average charge observed. No signals or very weak signals were observed at pH 3. It is unclear whether the oligonucleotides are degraded or insoluble under these conditions. Figure 2 shows an example of positive ion spectra for $tRNA^{fmet}$ (MW 24926.3) and $tRNA^{phenylalanine}$ (MW 24622.2). The positive and negative ion spectra of TAR 31-mer are compared in Figure 3.

Proteins and peptides usually contain a mixture of acidic and basic residues. Under basic solution conditions, the acidic residues are deprotonated and negative ion mass spectra are obtained with the maximum charge frequently correlating with the number of acidic residues. Likewise, under acidic solution conditions, the basic residues are protonated and positive ion spectra are obtained with the maximum charge frequently correlating with the number of basic residues. Recently, both positive and negative ion spectra of proteins have been observed over the pH range 3–10 [9, 10]. LeBlanc et al. [10] have proposed an explanation for the observation of positively charged proteins from basic solutions. In solution, complexes are formed between the proteins and nitrogen-containing bases. The complexes are desorbed into the gas phase and undergo CAD. The complex can dissociate by one of two ways depending on the relative proton affinities (PA) of the amino acid and the nitrogen-containing base. If the proton affinity of the amino acid is greater than the proton affinity of the nitrogen-containing base, then the proton will reside with the polypeptide (P) (eq 1). If the proton affinity of the amino acid is less than the proton affinity of the nitrogen-containing base, then the complex will dissociate as in eq 2:

$$[P\text{–}H\text{–}NH_3]^+ \xrightarrow{CAD} PH^+ + NH_3 \quad (1)$$

$$[P\text{–}H\text{–}NH_3]^+ \xrightarrow{CAD} P + NH_4^+ \quad (2)$$

Under conditions where CAD is minimized, complexes between proteins and nitrogen-containing bases have been observed. This supports the hypothesis that positive ions of proteins can arise from dissociation of the base/protein complex. The relative signal intensities of the positive protein ions correlate with the proton affinity of the nitrogen-containing bases and not with

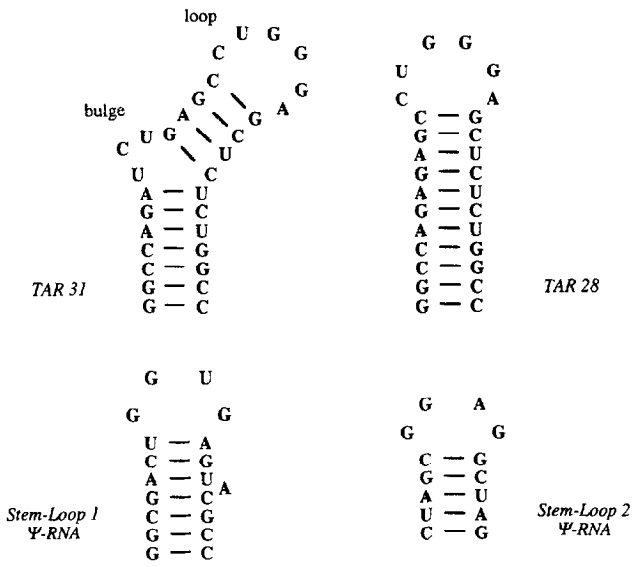

Figure 1. The sequences and secondary structures of selected RNA molecules. The positive charge may be located on the bulge and loop regions.

the pH of the solution, consistent with the proposal that gas phase reaction chemistry of the complex formed in the condensed phase is contributing to the ion formation process.

A similar proposal can explain the observation of the positive ion signals for DNA and RNA. Because our solutions contain 10 mM NH_4OAc, there is free NH_4^+ solution which can complex to the nucleotides and the phosphate groups of DNA and RNA. Because the proton affinity of the phosphate group is greater than that of ammonium ion, CAD of the ammonium ion/phosphate group complexes will neutralize the negative charge on the phosphate groups. Similarly, because the proton affinity of the bases is greater than that of ammonium ion [24–27], the bases will be protonated leading to a net positive charge on the DNA and RNA molecules.

In addition to 10 mM ammonium acetate solutions of DNA and RNA, positive ion ESI mass spectra were observed from 10 mM aniline, imidazole, diethylamine, and triethylamine. The proton affinities of aniline, imidazole, diethylamine and triethylamine are 209.49 kcal/mol, 223.50 kcal/mol, 225.91 kcal/mol and 232.19 kcal/mol, respectively [28]. Table 2 shows the maximum and average positive charge for $d(pA)_9$ and $d(pT)_9$ as a function of the proton affinity of these nitrogen-containing bases. In the case of $d(pA)_9$ (PA(dA) = 223.6 kcal/mol [25]), all four solutions gave positive ion mass spectra of approximately equal intensity. However, the maximum and averaged charge decreased as the proton affinity of the base approached the proton affinity of adenosine. Using gentle ESI interface conditions that minimize CAD, complexes between triethylamine and $d(pA)_9$ were observed. Thus, it is plausible that the protonated $d(pA)_9$ comes from the dissociation of a base/$d(pA)_9$ complex. In the case of $d(pT)_9$ (PA(dT) = 224.9 kcal/mol [25]), positive ion spectra were observed with all four solutions. The signal intensity decreased as the proton affinity of the

Table 1. The maximum and average positive charge observed by ESI-MS for RNA and DNA molecules

		Maximum charge (average charge)[a]		
RNA	MW (No. of bases)	pH 3	pH 6	pH 10
Ψ stem-loop 1	6131.8 (19)	–	4 (4.0)	4 (3.8)
Ψ stem-loop 2	4524.8 (14)	–	4 (3.5)	3 (3.0)
TAR 28-mer	9023.5 (28)	–	5 (5.0)	5 (4.8)
TAR 31-mer	9941.0 (31)	–	6 (5.4)	6 (5.2)
$tRNA^{phe}$	24622.2 (75)	na	9 (8.5)	na
$tRNA^{fmet}$	24926.3 (77)	na	10 (8.8)	na
DNA				
$d(pT)_3$	930.6 (3)	–	1 (1.0)	1 (1.0)
$d(pT)_6$	1843.3 (6)	–	2 (1.8)	2 (1.9)
$d(pT)_9$	2755.9 (9)	3 (2.1)	3 (2.0)	3 (2.1)
$d(pA)_9$	2836.9 (9)	–	3 (2.3)	3 (2.4)
$d(pC)_9$	2620.9 (9)	3 (2.9)	3 (2.8)	3 (2.9)
$d(pT)_{12}$	3668.5 (12)	–	3 (2.9)	3 (2.9)
$d(pT)_{18}$	5493.8 (18)	–	4 (3.8)	4 (3.9)
$d(pC)_{18}$	5224.8 (18)	5 (4.0)	5 (4.0)	4 (4.1)
Sac I (NP)[b]	2410.6 (8)	na	3 (2.1)	na
Sac I (P)[b]	2490.6 (8)	na	3 (2.3)	na
Eco R I (A)[c]	6827.4 (22)	–	5 (4.5)	5 (4.8)
Eco R I (B)[c]	6796.4 (22)	–	5 (4.4)	5 (4.8)

[a]na: not applicable; dashed line (–) indicates that no signal was observed. [b](NP): nonphosphorylated, (P): phosphorylated. [c](A) 5′ to 3′ strand of Eco R I; (B) 3′ to 5′ strand of Eco R I.

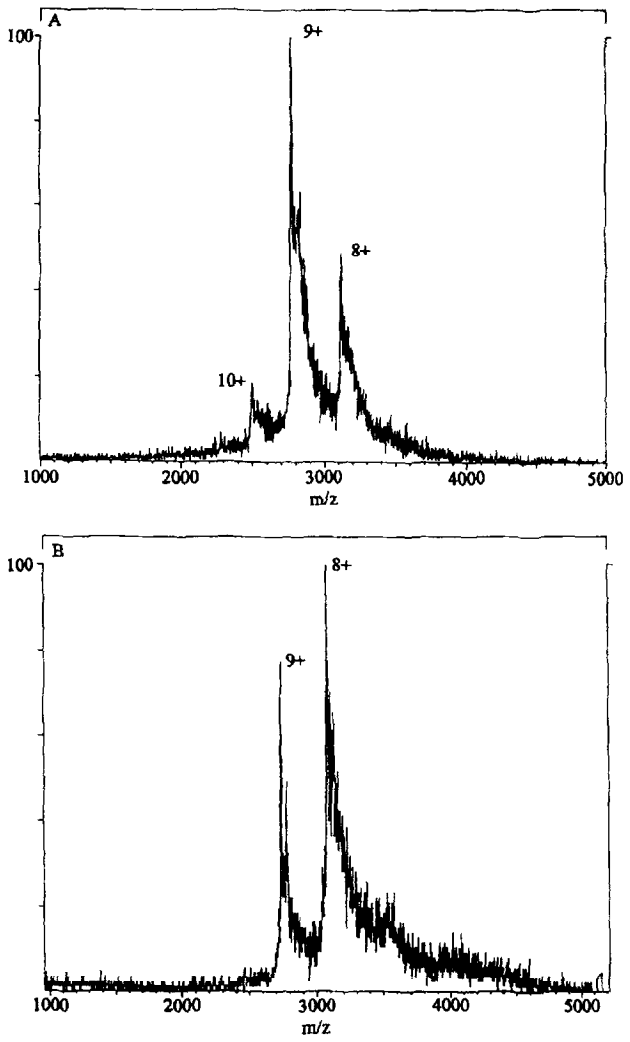

Figure 2. (a) Positive ion ESI mass spectra of $tRNA^{fmet}$ which shows a maximum charge of 10. The measured mass is 24961.8 ± 5.7. Sequence: $_pCGCGGGGs^4UGGAGCAGCCUGGDAGCUCGU-CGGGCmUCAUAACCCGAAGm^7GUCGUCGGT\Psi CAAAUCC-GGCCCCCGCAACCA_{OH}$ [31]. (b) Positive ion ESI mass spectra of $tRNA^{phenylalanine}$ which shows a maximum charge of 9. Sequence: $_pGCGGAUUUAm^2$ $GCUCAGDDGGGAGAGCm^2_2GC-CAGACmUGmAAYA\Psi m^5CUGGAGm^7$ $GUCm^5CUGUGT-\Psi CGm^1AUCCACAGAAUUCGCACCA$ [32]. The measured mass (24665.8 ± 7.5) is more consistent for $tRNA^{phenylalanine}$ lacking the terminal A_{76} [32]. In both examples, a 5 μM tRNA solution in 5 mM ammonium acetate [pH 6, 40% methanol (*v*/*v*), 0.2 mM CDTA] was used.

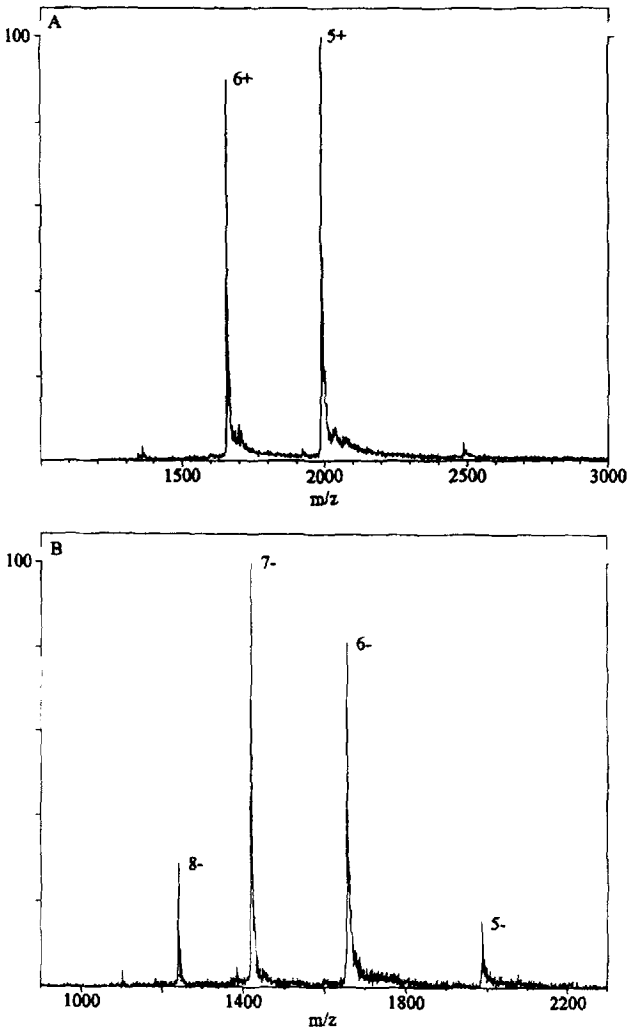

Figure 3. (a) Positive ion and (b) negative ion ESI mass spectra of TAR 31-mer, showing protonated and deprotonated ions, respectively. In both cases, a 10 μM TAR 31-mer solution in 10 mM ammonium acetate [pH 6, 10% methanol (*v*/*v*), 0.25 mM CDTA] was used. An accurate mass is obtained from both spectra (positive ion ESI-MS: 9940.6 ± 0.8; negative ion ESI-MS: 9941.6 ± 0.9).

nitrogen-containing base increased. In addition, imidazole, diethylamine and triethylamine primarily gave adduct ions and adducts from sodium salt impurities. An accurate mass could not be determined from these spectra. Furthermore, at higher ΔV_{TS}, the triethylamine adducts of $d(pT)_9$ appear to dissociate such that the proton stays with triethylamine and the oligonucleotide signal is significantly reduced. The only ions observed are sodium salt adducts of $d(pT)_9$, most likely reflecting the greater stability of these gas phase ions; no protonated $d(pT)_9$ ions are observed. Thus, the protonated oligonucleotide will less likely be observed when the proton affinity of the base is greater than that of the nucleotide.

Lowering the dielectric constant of the solution should increase complex formation between the ammonium ion and DNA/RNA molecules and hence increase the signal intensity. Increasing the amount of methanol from 10% to 50% in the 10 mM NH_4OAc solution decreased the dielectric constant of the solution and resulted in a marginal increase in signal intensity. When the DNA and RNA molecules are run under the same tuning conditions but in unbuffered water/methanol solutions, no positive ion signals (or very weak signals) are observed. This indicates that the ammonium ion (or a nitrigen-containing base) is necessary for the formation of positive ions of DNA and RNA.

There is weak evidence that secondary structure may possibly contribute to positive charging. For the

Table 2. The maximum and average positive charge observed by ESI-MS for $d(pA)_9$ and $d(pT)_9$ as a function of the proton affinity of the bases

Base	PA (kcal/mol)	Max. (ave.) charge for $d(pA)_9$	Max. (ave.) charge for $d(pT)_9$
Ammonia	203.99	3 (2.2)	3 (2.0)
Aniline	209.49	3 (2.2)	2 (2.0)
Imidazole	223.50	2 (2.0)	2 (1.8)
Diethylamine	225.91	2 (2.0)	2 (1.5)
Triethylamine	232.19	2 (1.8)	2 (1.4)

small RNAs (i.e., Ψ stem-loop 1, Ψ stem-loop 2, TAR 28-mer, and TAR 31-mer) in an ammonium acetate solution, the maximum charge appears to correlate with the number of unpaired A, C, G moieties (i.e., those found in the loop and bulge regions). The unpaired U bases are not expected to be protonated because the proton affinity of U is less than those of A, C, G by about 10 kcal/mol [24–27]. In the double stranded region, the bases are paired and are not expected to form complexes with the ammonium ion. The phosphate groups on the paired bases still need to be protonated by forming a complex with the ammonium ions. TAR 28-mer lacks the UCU bulge found in TAR 31-mer. Based on the above proposed correlation, the UCU bulge should only carry one charge on C. Thus, the maximum charge on the TAR 28-mer should be one less than the maximum charge on the TAR 31-mer. The experimentally observed maximum positve charge on TAR 28-mer is 5, which is one less than the maximum charge on TAR 31-mer (a charge of 6+) and in agreement with our prediction. However, the correlation of unpaired A, C, G moieties with maximum charge does not hold for the tRNAs. Less charging than expected is observed. More systematic studies are needed to determine the contribution of secondary structure to gas phase charging.

For homo-oligonucleotides of the same length, neither the average charge nor the maximum charge correlates with the proton affinities of the individual deoxyribonucleotides. The proton affinities of dA, dC, and dT are 233.6, 233.2, and 224.9 kcal/mol, respectively [25]. The average charge increases in the series $d(pT)_9$, $d(pA)_9$ and $d(pC)_9$, while the maximum charge is the same for all three (Table 1). Based on their respective proton affinities, the average charge for $d(pA)_9$ should be greater than or nearly equal to that of $d(pC)_9$. Charge repulsion does not seem to be the limiting factor for the maximum or average charge on an oligonucleotide. From the maximum charge on $d(pT)_3$, it appears that every three bases can hold one charge and a maximum charge of 6 would be predicted for $d(pT)_{18}$. However, a maximum charge of only 4 is observed. Another factor besides charge repulsion must limit the maximum charge on the oligonucleotides. The lower than expected charging may be consistent with the CAD mechanism. The ESI interface conditions are slightly harsher than typically used for similar sized peptides in order to dissociate the oligonucleotide/nitrogen-base complex. Higher charged ions would be more susceptible to dissociation at higher ΔV_{TS} (higher laboratory-frame collision energies).

For the shorter length oligonucleotides at pH 6, there appears to be a linear correlation between the observed average charge and the number of bases (n) (Figure 4). The R^2 coefficient is 0.96 and the least squares fit gives the following equation: average charge = 0.15(n) + 0.99. However, the plot predicts an average charge of 12.4 and 12.5 for $tRNA^{phenylalanine}$ and $tRNA^{fmet}$, respectively; an average charge of 8.5 and 8.8 are experimentally observed for $tRNA^{phenylalanine}$ and $tRNA^{fmet}$, respectively. Again, the higher collision energies in the ESI interface necessary to observe positive ions for the larger oligonucleotides may not be favorable conditions for the survival of the higher charged ions.

Conclusions

Positive ion ESI mass spectra were observed for a variety of DNA and RNA molecules. In solution, all oligonucleotides have a net negative charge and can form noncovalent complexes with positive counter ions such as ammonium ions. Desorption of these noncovalent complexes into the gas phase followed by CAD leads to positively charged DNA, RNA, and tRNA molecules by neutralization of the phosphate groups on the sugar backbone and protonation of some of the bases. A similar scheme was proposed for the observation of positively charged proteins under basic solution conditions [10] and for charge state reduction of

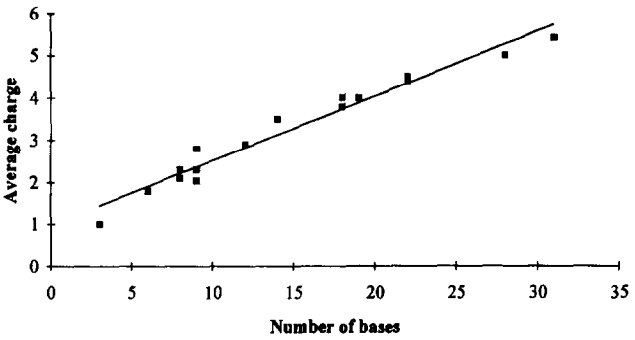

Figure 4. A plot of the average charge observed vs. the number of bases. The tRNA molecules are not included in the plot.

oligonucleotide negative ions with nitrogen-containing bases [29, 30]. Thus, oligonucleotide ions can be observed using positive ion ESI under conditions that favor dissociation of the DNA/RNA-adduct complex.

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