Comparison of A + T-rich oligonucleotides with and without self-complementary sequence using ion-pair reversed-phase high-performance liquid chromatography/tandem electrospray ionization mass spectrometry

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Both A + T-rich oligonucleotides with and without self-complementary sequences were analyzed using ion-pair reversed-phase liquid chromatography/electrospray ionization mass spectrometry (IP-RP-HPLC/ESI-MS) by tryethylammonium acetate (TEAA) and hexafluoroisopropanol (HFIP) buffer systems to study the characteristics of their retention behavior and electrospray ionization tandem mass spectrometry (ESI-MS/MS) response. The results indicated that the chain length had the same effect on the retention of A + T-rich oligonucleotides in both of TEAA and HFIP buffer systems but the sequence had a different impact on the retention in the two buffer systems. A + T-rich oligonucleotides with a self-complementary sequence were much shorter than those without in the TEAA buffer system whereas a slight difference was observed in the HFIP buffer system. Similar total ion current (TIC) intensity was observed both in oligonucleotides with or without self-complementary sequence. The opposite trend of a change in the TIC intensities with increasing chain length were observed in both the TEAA and HFIP buffer systems. A lower charge state was predominant in the TEAA buffer system whereas a higher charge state was mainly distributed in the HFIP buffer system. The oligonucleotides without self-complementary sequences had a higher charge state than those with self-complementary sequences. A- and T- are more esily formed at a higher charge state whereas the sequence fragments will be formed more easily at a lower charge state in both A + T-rich oligonucleotides with and without self-complementary sequences.

Keywords: A + T-rich oligonucleotides, self-complementary sequence, ion-pair reversed-phase high-performance liquid chromatography, electrospray ionization mass spectrometry, charge state

Introduction

A + T-rich oligonucleotides have had more important functions in the A + T-rich region which have been found in recent research works.[1–4] The human genome contains a unique class of domain, referred to as AT islands (AT minisatellites). They play an important part in, for example, simple sequence repeat replication origins, fragile site genesis, bending, HMG interaction sites etc. AT islands are site-instable in the genome of cancer cells and targets for extremely lethal AT-specific drugs.[5] Furthermore, the A + T-rich sequence has often been composed of the hyperactivity promoter that induced mutation.[6] Another significant aspect is that more and more evidence has indicated that some hereditary diseases are related to the A + T-rich region.[7,8] Some potent genetoxic chemical compounds will bind to the A + T-rich region more easily than other regions in DNA, which would explain why the AT-rich sequence easily mutates.[9,10] On the other hand, recent research indicates that the significance of AT islands as potential targets for chemotherapeutic intervention stems from two aspects, which are that AT islands are inherently unstable and have affinity to genomic DNA. So, it may be a

factor in cancerous phenotype and a target for the extremely potent DNA-alkylating antitumor drugs, as well as an optimal target for proteins to recognize AT-rich regions.

Secondary structure is one of the important properties of A+T-rich oligonucleotides, especially A+T-rich oligonucleotides with a self-complementary sequence. Furthermore, the secondary structure will not only form in dsDNA but also in oligonucleotides, even at those rather short length scales of less than 10 bases.[1] The secondary structure also exists in the oligomers with alternating base sequences, ATAT, pd (AT) 12 and d (ATATAT).[11,12] It is well known that the secondary structure is a very important property of DNA and it plays a key role in biology in such areas as recombination processes transposition and gene expression.[1,13] The study of secondary structures has been focused mainly on DNA, RNA and GC-rich oligonucleotides using capillary electrophoresis, denaturing high-performance liquid chromatography and atomic force microscopy.[14–16] However, few investigations have been reported about the A+T-rich oligonucleotides with and without self-complementary sequence using reversed-phase liquid chromatography/electrospray ionization mass spectrometry (IP-RP-HPLC/ESI-MS).[17]

IP-RP-HPLC/ESI-MS is an important technique for the characterization of thermally unstable and polar biopolymers such as proteins and nucleic acids. Compared with traditional methods of oligonucleotide analysis, high-performance liquid chromatography/tandem electrospray ionization mass spectrometry (HPLC/MS-MS) offered not only a higher level of selectivity and specificity for oligonucleotide mixtures but also the advantage of providing reliable structural information of oligonucleotides.[18–21]

HPLC/MS-MS mobile phase systems affect not only the retention time but also the ESI/MS behavior. Triethylammonium acetate (TEAA) and triethylamine-hexafluoroisopropanol (TEA-HFIP) were commonly used as ion-pairing buffers in the analysis of DNA.[21] However, these two buffer systems may have different effects on the retention mechanism of oligonucleotides on HPLC, i.e. the hydrophobic and charge–charge interactions in the system contribute to the retention of oligonucleotides in many different ways. TEAA is a weak ion-pairing system that does not completely eliminate the impact of the oligonucleotide sequence on its retention, whereas the HFIP buffer can practically eliminate the impact of the oligonucleotide sequence on retention and effectively disrupts the G-rich oligonucleotide secondary structure intra- and intermolecular complexes.[22]

More recently, we have studied the characteristics of G-rich and T-rich oligonucleotides and analyzed the DNA methylation by IP-RP-HPLC/ESI-MS.[23,24] In the present study, six pairs of A+T-rich oligonucleotides, with or without secondary structures, were analyzed using IP-RP-HPLC/ESI-MS to compare their characteristics of retention time, ESI-MS response, charge–state distribution and product ion formation at different collision energies.

Experimental

Chemicals and materials

Oligonucleotides were obtained from Asia Biochemistry (Shanghai, China) and used as received. The sequences

Table 1. Characteristics of oligonucleotides used in the experiments.

Sample no.	Sequence	Moleculer weight (Da)		Length	A+T content%
		Theoretical	Experimental		
Group 1					
AT-1	5′-CATATATG-3′	2408.7	2408.1	8	75.0
AT-3	5′-CATATATATG-3′	3026.1	3025.6	10	75.0
AT-5	5′-CATATATATATG-3′	3643.5	3643.9	12	80.0
AT-7	5′-GCATATATATATATATATGC-3′	6114.1	6114.3	20	80.0
AT-9	5′-GCATATATATATATATATATATATATATGC-3′	9201.2	9202.3	30	83.3
AT-11	5′-GCCATATATATATATATATATATATATATATATATATGGC-3′	12289.2	12288.2	40	83.3
Group 2					
AT-2	5′-ACTTTGAA-3′	2408.7	2407.8	8	80.0
AT-4	5′-AACTTTTGAA-3′	3026.1	3026.2	10	80.0
AT-6	5′-AACTTTTTGAAA-3′	3643.5	3643.1	12	86.7
AT-8	5′-AAGACTTTTAATTTTAAGAC-3′	6114.1	6115	20	86.7
AT-10	5′-AAAGAAATTTTTACTTTTTGTTTAAACAAA-3′	9201.2	9201.7	30	85.0
AT-12	5′-AAAGAAACTTTTTAATTTTTACATTTTTATTAAAGAAGAC-3′	12289.2	12289.3	40	85.0

investigated are listed in Table 1. Triethylamine (TEA, 99.5%), glacial acetic acid (HOAc 99.99%), HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Water was purified with an Elix-Milli-Q system (Millipore Corp., Bedford, MA, USA). 1,1,1,3,3,3-hexafluoro-2-propanol was obtained from Dupont (Delaware, USA).

HPLC mobile phase preparation

The 100 mM triethylammonium acetate buffer, pH 7, was prepared by mixing 7.2 mL TEA and 2.98 mL acetic acid in 400 mL water and then carefully adjusted to pH 7 using TEA or acetic acid. After adjusting the volume to 500 mL, the final concentration of triethylammonium acetate is 100 mM.

The 400 mM hexafluoroisopropanol–16 mM triethyl-ammonium buffer, pH 7.7, was prepared by dissolving 22 mL HFIP in 400 mL water and then slowly titrating with 1.2 mL TEA.

All mobile phases were filtered through 0.22 μm film before being used in the HPLC analysis.

Instrumental analysis

All samples were analyzed using an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). The liquid chromatograph was equipped with an on-line vacuum degassing system, a quaternary pumping system, an autosampler and a variable wavelength detector. The chromatographic separation was performed on a C18 column (150 × 3.0 mm i.d., 3.0 μm particle size, Zorbax 300SB, Agilent Technologies, Palo Alto, CA, USA) at room temperature. Three mobile phases at the flow-rate of 300 μL min^{-1} were used in the analysis and the conditions can be seen in the figure captions. The concentration of a single oligonucleotide used in the experiments was 0.2 nmol μL^{-1}. The injection volume was 1 μL and UV detection was carried out at the 260 nm wavelength. The LC effluent was introduced into a Turbon IonSpray interface without splitting. Electrospray mass data were acquired in the negative-ion mode with a spray voltage of –4 kV and declustering potential of –100 V. The source temperature was 400°C. Nitrogen was used as the curtain gas (setting 16), nebulizer gas (setting 20) and turbo gas (setting 20). Tandem mass spectrometry (MS/MS) was performed using nitrogen as collision gas (CAD gas setting 8). The mass spectrometer was operated at unit mass resolution for both Q1 and Q3. The data were acquired using Sciex Analyst software, version 1.3.1 (Applied Biosystems, Foster City, CA, USA).

Results and discussion

Retention behavior of both A + T-rich oligonucleotides with and without self-complementary sequences on IP-RP-HPLC

Size is a desirable factor in chromatographic separation of DNA, because size, usually expressed as the number of base pairs, is basic and necessary information for the identification of a DNA molecule. Besides size, however, there are other important molecular properties and structural features such as shape, conformation, curvature and sequence variations, which are responsible for the biological function of a DNA molecule and directly related to its specific base sequence. The base sequence, therefore, is another helpful separation criterion for the fractionation of oligonucleotides.[25] In the present study, two groups of A + T-rich oligonucleotides, with or without self-complementary sequences and of identical size and base composition, were analyzed using IP-RP-HPLC/ESI-MS to characterize the sequence of A + T-rich oligonucleotide contributions to the retention time.

Although the introduction of ion-pairing buffers is very helpful for improving separation selectivity for resolving oligonucleotides, and achieves a regular retention of oligonucleotides according to their size,[26] different ion-paring agent systems may have significant effects on the retention of the oligonucleotides with different sequences despite the same base composition, especially those with a secondary structure. In our study, we have found an interesting phenomenon that the retention times of A + T-rich oligonucleotides with self-complementary sequences were much shorter than those with optional sequences in the TEAA buffer system whereas slight differences were observed in the HFIP buffer system [Figure 1(a) and 1(b), respectively].
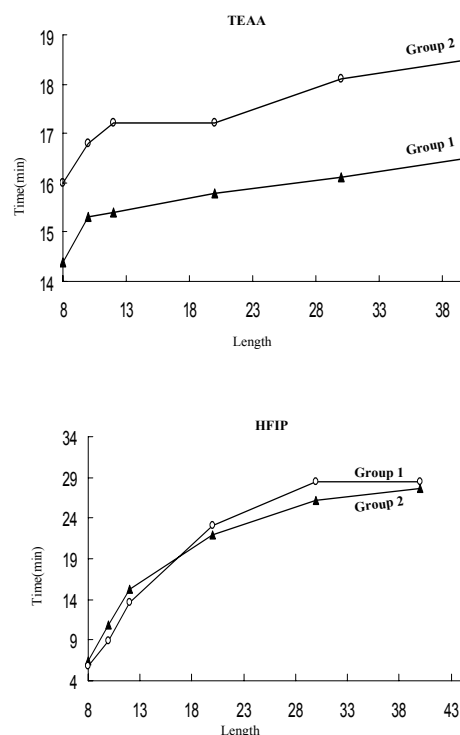

Figure 1. Retention time of two groups of oligonucleotides with the same length and base composition but different sequence in the TEAA and HFIP buffer systems. Group 1 implicates the oligonucleotides with odd numbers (AT-1, AT-3…) and group 2 represents the oligonucleotides with even numbers (AT-2, AT-4…).
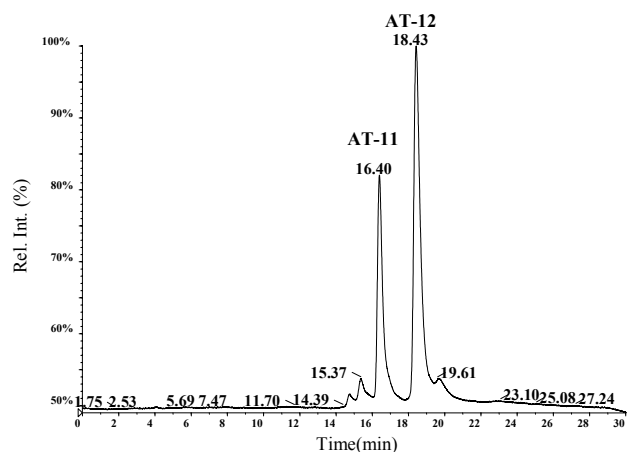
Figure 2. The LC spectrum of AT-11 and AT-12 in the TEAA buffer system.
AT-11: 5′-GCCATATATATATATATATATATATATATATATATGGC-3′.
AT-12: 5′-AAAGAAACTTTTTAATTTTTACATTTTTATTAAAGAAGAC-3′.

As an example, Figure 2 gives the HPLC chromatogram of a pair of A+T-rich oligonucleotides eluted by the TEAA buffer system. It is clear to see that oligonucleotides with a self-complementary sequence were eluted faster than those with optional sequences. This result indicated that the base sequence and the ion-paring agent might play important roles in the retention behavior of oligonucleotides. As reported by other research, oligonucleotides with a self-complementary sequence will easily form the secondary structure so that its conformation will tend to be a globular shape while the shape of the oligonucleotide with no secondary structure is linear.[13] The self-complementary oligonucleotides with a globular shape would decrease the surface area for the hydrophobic interactions of nucleobases with reversed-phase sorbent, so the retention of oligonucleotides with a self-complementary sequence will be eluted faster than that without a self-complementary sequence in the TEAA buffer system. This also provides direct evidence that the HFIP, rather than the TEAA, was an efficient denaturant for eliminating the impact of the oligonucleotide sequence on its retention. Hence, the retention of oligonucleotides in the HFIP buffer system was mainly determined by charge-to-charge interactions of negatively-charged phosphate groups with the ion-pairing agent adsorbed on the stationary phase and the separation mechanism of oligonucleotides was performed mainly according to their base composition. Therefore, no significant difference in the retention times between the oligonucleotides with or without self-complementary sequences was observed in the HFIP buffer system.

Characterization of both A+T-rich oligonucleotides with and without self-complementary sequence by IP-RP-HPLC/ESI-MS/MS

It is well known that the ions in ESI are generated by applying a high voltage to the sprayer through which a solution containing analyte is infused. Several factors would influence the ESI response of an analyte in a mass spectrum. A significant factor is the composition of the electrospray solvent. During ionization, the used solvents will result in different surface charge density on the droplet and the charged offspring droplets, which has a crucial effect on the subsequent formation of the analyte ions. The second factor is the quantity and type of the sample, mainly referred to as the surface activity and the free energy of the analyte. The surface activity affects the nature of ion evaporation from a charged droplet and the free energy is required to remove the ion from the droplet. The ESI performance of the analyte will also depend upon other factors such as ESI voltage, nebulizer gas, heater gas flow and temperature, which can be controlled by the mass spectrometrist. Many researchers have illustrated that different solvents and buffers would result in dramatically different mass signal response and so the choice of a compatible mobile phase is very important in obtaining both a good resolution of the analytes and a higher ESI response in HPLC analysis. In our previous work, the characteristics of G-rich and T-rich oligonucleotides were investigated using IP-RP-HPLC/ESI-MS/MS.[23] However; very little research has been focused on the characteristics of A+T-rich oligonucleotides by IP-RP-HPLC/ ESI-MS/MS.

In the present study, the ESI-MS responses of A+T-rich oligonucleotides were investigated on a triple quadrupole mass spectrometer in negative-ion mode using TEAA and HFIP buffer systems. From Figure 3, we can see that the total ion current (TIC) intensities of A+T-rich oligonucleotides
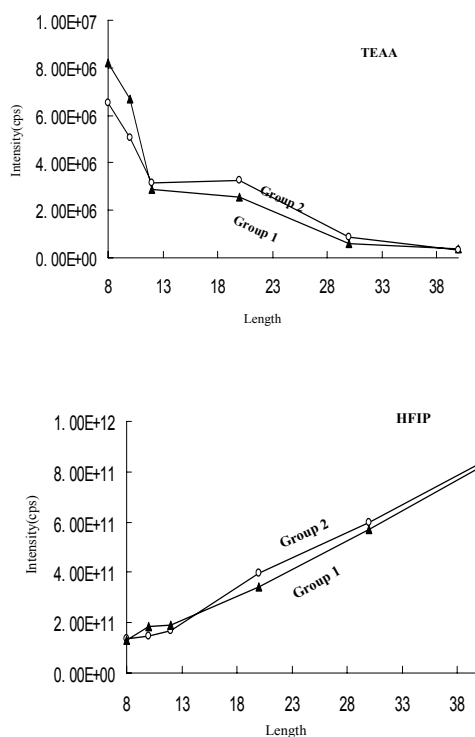

Figure 3. Comparison of TIC intensity of two groups of oligonucleotides with the same length and base composition but different sequence in the TEAA and HFIP buffer systems.

with or without self-complementary sequences are similar. This phenomenon could be well understood because no secondary structure can be formed under a high temperature of 400°C. Actually, elevated temperature is one of the common methods for the purification and analysis of oligonucleotides to eliminate the impact of secondary structure.[22,27] Figure 3 also shows the relationship between TIC intensity and the chain length of A + T-rich oligonucleotides. Clearly, an opposite trend of TIC intensity change with the chain length was observed in the TEAA and HFIP buffer systems, i.e. the TIC intensities of oligonucleotides decreased with the increase in chain length in the TEAA buffer system, whereas the TIC intensities increased with the increase in the chain length in the HFIP buffer system. Furthermore, the TIC intensity obtained from the HFIP buffer system was much higher than that obtained from the TEAA buffer system. The reason for this may be that HFIP is a weaker acid than HOAc, so the linkage of the oligonucleotides with HOAc would be much stronger than that of the HFIP. Since weaker bonding would be beneficial to being broken to form more analyte ions in the ESI process, the ESI-MS response of oligonucleotides in the HFIP buffer system was much stronger than that in the TEAA buffer system. Another reason for the higher ESI response obtained in the HFIP buffer system may be that the boiling point of HFIP was much lower than that of acetate (HFIP = 59°C, TEA = 89°C, HOAC = 118°C, respectively). During the spraying, HFIP was easily removed from the solution so the TIC intensity was much stronger than that in the TEAA buffer system. On the other hand, we know that the ESI response was not only related to the analyte property but also to the solvent used. It can be expected that the hydrophobicity of the A + T-rich oligonucleotide would increase with an increase in the chain length. According to the theory obtained by Cech *et al.*,[28] in which the retention time of peptides was used to predict the ESI response and the ESI response was increased with the increasing non-polar character, one can speculate that the ESI response should be increased with the increasing chain length. However, acetic acid seems to be the principal component in the TEAA buffer responsible for ion suppression.[29] If ion suppression of abundant acetic acid existed in the TEAA buffer system, it would be increased with the increasing chain length and much higher than the enhancement of ESI response due to the hydrophobicity. So, the ESI-MS signal intensities of A + T-rich oligonucleotides decreased with the increasing chain length. Due to HFIP acid being much weaker than acetic acid (pKa value HFIP = 9.2, HOAc = 4.76, respectively), the ion suppression of HFIP is much lower than the enhancement of the hydrophobicity, so the ESI-MS response of A + T-rich oligonucleotides increased with the increase in chain length. This result indicated that the sequence is not the predominant impact factor on TIC intensity compared with chain length and hydrophobicity.

In addition, the TEAA and HFIP buffer systems led to different charge-state distribution of A + T-rich oligonucleotides on the ESI-MS spectrum. Figure 4 shows
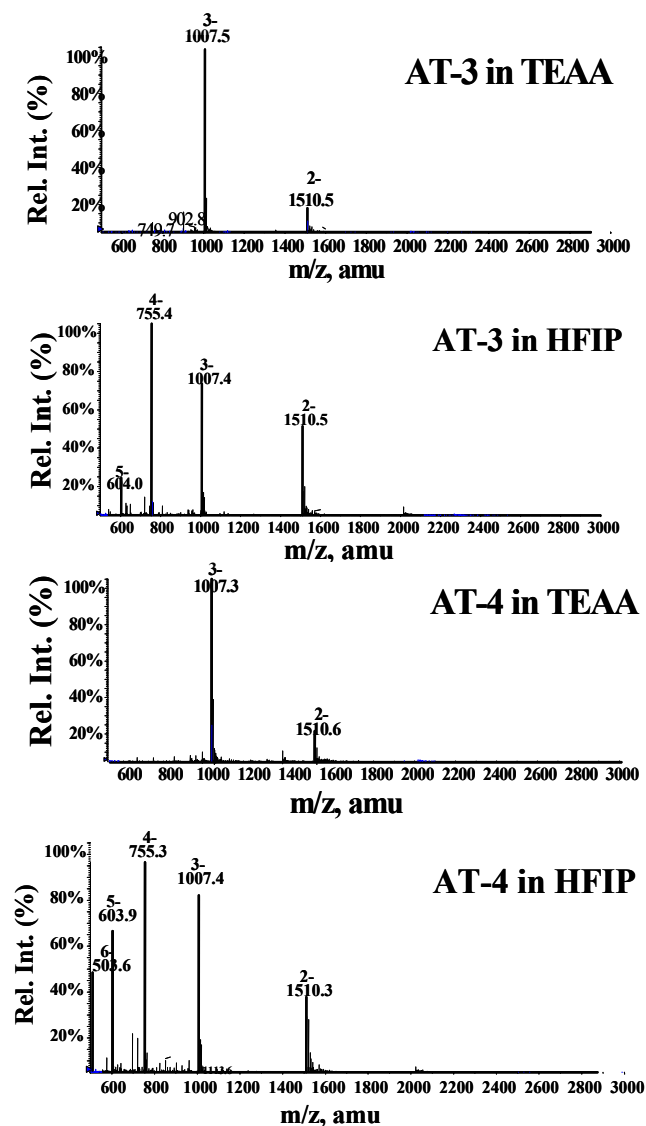

Figure 4. The charge state of AT-3 and AT-4 in HFIP and TEAA buffers. AT-3: 5′-CATATATATG-3′ AT-4: 5′-AACTTTTGAA-3′.

the charge-state distribution of oligonucleotides with and without self-complementary sequences eluted by two buffers. It can be observed that the lower charge state of 3- was predominant in the TEAA buffer systems whereas in the HFIP buffer system the charge–state distribution typically covered a range from 2- to 6-, predominant at 4-. The longer the length, the more the charge state was found in our experiment (data not shown). So, the HFIP buffer system can produce more abundant multiple charge-state distributions than the TEAA buffer system. These results can also be explained on the basis of solution and gas-phase acid-based equilibrium.[30] The more acidic the solution, the more likely the acids will donate protons to oligonucleotide anions and reduce the charge states of oligonucleotides. Since HFIP is a weaker acid than HOAc, a higher charge state was observed in the HFIP buffer system.
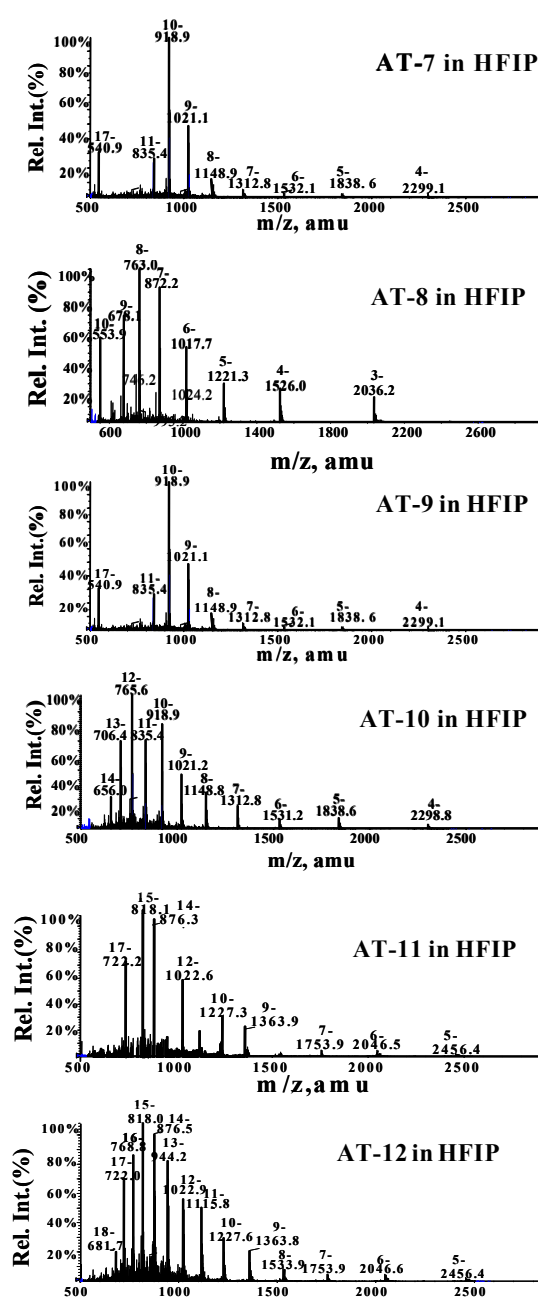
Figure 5. The charge state distribution of AT-7, 9, 11 and AT-8, 10, 12 in the HFIP buffer system.

Figure 6. The MS/MS spectra of oligonucleotides AT-5 and AT-6 at three charge states. AT-5: 5′-CATATATATATG-3′, AT-6: 5′-AACTTTTTGAAA-3′

Interestingly, the sequence will affect the charge-state distribution of oligonucleotides. Figure 5 shows the charge-state distribution of three oligonucleotide pairs. From the figure we can see that the oligonucleotide without a self-complementary sequence has a higher charge state. Similar results were observed with other oligonucleotides in our experiments (data not shown). Maybe the oligonucleotide with a self-complementary sequence has a stronger bond force in the intermolecular and a hydrogen bond in the outer-molecular.[1,31] The oligonucleotide with a self-complementa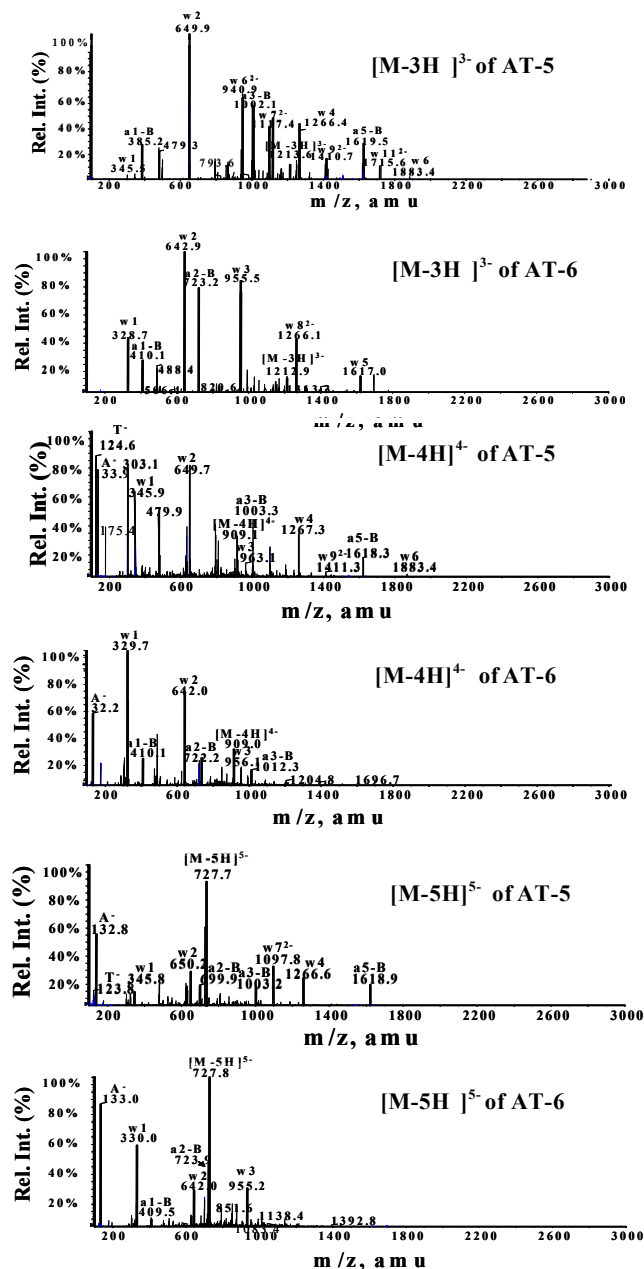ry sequence would be more difficult to spray and ionize than one without a self-complementary sequence, so that fewer charge-state ions were formed in the oligonucleotide with the self-complementary sequence than that with the optional sequence. To our knowledge, the effect of the sequence on charge-state distribution has not been reported in the literature up to this date. This is the first study to learn the charge-state distribution of oligonucleotides with the same base composition but a different sequence.

The A + T-rich oligonucleotides were also analyzed using MS/MS in order to investigate whether the sequence would affect the product-ion distribution from the parent molecular

Table 2. The fragments of AT-7 at −35 eV collision energy for different charge states.

Ion type	Relative abundance				
	6-	5-	4-	3-	2-
T-	100	100	65.7	nd	nd
A-	78.6	90.2	42.8	nd	nd
a1-B	5.7	17.5	39.8	42.6	37.2
w1	10.3	39.6	100	73.3	82.4
w2	6.8	21.8	79.6	100	100
w3	2.9	9.1	33.7	69.7	95.4
a8-B	0.1	0.9	1.2	29.8	48.6

nd (not detected)

Table 3. The fragments of AT-8 at −35 eV collision energy for different charge states.

Ion type	Relative abundance				
	6-	5-	4-	3-	2-
T-	13.5	18	2.78	nd	nd
A-	100	100	53.4	nd	nd
a1-B	2.2	8.1	20.1	22.9	22.5
w1	8.1	27.6	100	23.7	46.8
w2	1.3	9.5	69.6	100	100
w3	0.1	1.6	13.8	79.4	95.4
a8-B	nd	nd	0.2	38.1	38.3

nd(not detected)

ion with an identical charge. The collision energy was set to maintain a relative abundance of the precursor ions. Figure 6 shows the MS/MS spectra of oligonucleotides AT-5 and -6 in charge states from $[M−3H]^{3−}$ to $[M−5H]^{5−}$, obtained with collision energies of –40, –30 and –20 eV, respectively. In the AT-6 oligonucleotide, the T was linked together. Therefore the predominant product ions of $[M−3H]^{3−}$ were w1, w2, w3, a1-B, a2-B and a3-B. When the precursor ions were $[M−4H]^{4−}$ and $[M−5H]^{5−}$, A- was added to the main product ions. However, in AT-5 oligonucleotide with a self-complementary sequence, the main product ions of $[M−3H]^{3−}$ were w2, w4, w7, w9, a1-B, a3-B and a5-B. When the precursor ions were $[M−4H]^{4−}$ and $[M−5H]^{5−}$, A- and T- were added to the main product ions. This indicated that the structure (sequence) of oligonucleotides would affect the product ion distribution. This result accorded well with the observation obtained by Luo *et al.*[32]

The multiply-charged nature of the parent ion introduces complexity in the mass spectrum of the product ions.[31] The MS/MS spectra of all A + T-rich oligonucleotides with different charge states were compared in order to understand which charge state is compatible for forming the sequence fragments. To simplify, only Tables 2 and 3 give the details of the fragments produced by different charge states at the same collision energy of AT-7 and AT-8. From the tables, we can see that A- is easier to form at a higher charge state than that of T- in the oligonucleotide without a self-complementary sequence, which was similar to the results obtained by McLuckey *et al.*, in which they found that the base ions formed will follow the order: A > G > T > C.[33] Meanwhile A- and T- could easily be formed at a higher charge state in the oligonucleotide with a self-complementary sequence. It was also found that, at the same collision energy, the sequence fragments can easily be formed at the lower charge state. However, the precursor ion with the higher charge state needed a lower collision energy to form the sequence fragments. The reason may be that the energy surface and energy for break-up of the proton-bound intermediates is higher for the higher charge–state oligonucleotides.[33]

The collision energy also affected the fragmentations of A + T-rich oligonucleotides with or without self-complementary sequences. According to the results of McLuckey and Goudarzi, the energy associated with the loss of a charged base was increased at a multiply-charge state because the proton affinity of A and T is smaller than that of G and C.[34] Figure 7 shows the relative abundance of A- and T- bases with increasing collision energy. The relative abundance of A- and T- from the oligonucleotide with a self-complementary sequence was similar except at CE = −60 eV, where the relative abundance of the T- base was much higher than that of A-. On the other hand, for the oligonucleotide with
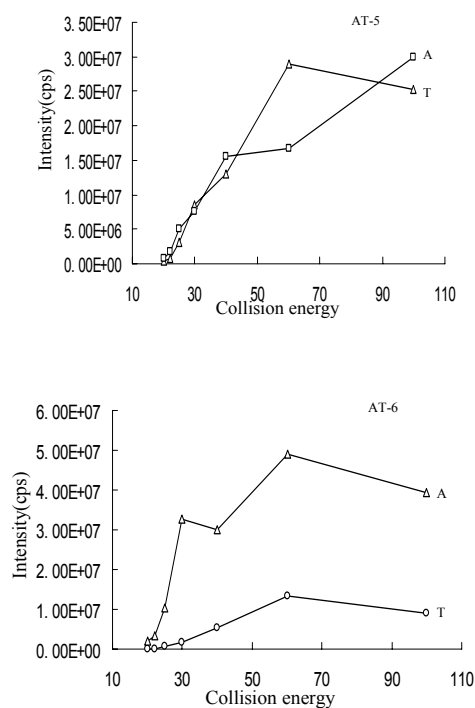
Figure 7. The intensities of A- and T- fragment of AT-5 (5′-CATATATATATG-3′) and AT-6: (5′-AACTTTTTTGAAA-3′) produced at different collision energy.

a discretional sequence, the relative abundance of the A-base was higher than that of T-. Similar results were also found in other experiments (data not shown). So, the probability of losing A or T is affected by collision energy and the sequence.

Conclusions

The retention times of A + T-rich oligonucleotides on IP-RP-HPLC were increased with an increase in chain length for both the TEAA and HFIP buffer systems. However, the sequence had a different effect on the retention time in the TEAA and HFIP buffer systems. Opposite trends of the TIC intensity change with increasing chain length were observed in the TEAA and the HFIP buffer systems. The TIC intensity obtained from the HFIP buffer system was much higher than that obtained from the TEAA buffer system. The TIC intensities of A + T-rich oligonucleotides with or without self-complementary sequences were similar. Different charge-state distribution was observed in both the TEAA and HFIP buffer systems and the oligonucleotide without a self-complementary sequence had more charge state. It was proved that sequence, collision energy and charge state may have an important significance in the interpretation of the MS/MS spectra.

Acknowledgment

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