RESEARCH ARTICLE

Tandem mass spectrometric sequence characterization of synthetic thymidine-rich oligonucleotides

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Abstract
Tandem mass spectrometry (MS/MS) can provide direct and accurate sequence characterization of synthetic oligonucleotide drugs, including modified oligonucleotides. Multiple factors can affect oligonucleotide MS/MS sequencing, including the intrinsic properties of oligonucleotides (i.e., nucleotide composition and structural modifications) and instrument parameters associated with the ion activation for fragmentation. In this study, MS/MS sequencing of a thymidine (T)-rich and phosphorothioate (PS)-modified DNA oligonucleotide was investigated using two fragmentation techniques: trap-type collision-induced dissociation (“CID”) and beam-type CID also termed as higher-energy collisional dissociation (“HCD”), preceded by a hydrophilic interaction liquid chromatography (HILIC) separation. A low to moderate charge state (−4), which predominated under the optimized HILIC-MS conditions, was selected as the precursor ion for MS/MS analysis. Comparison of the two distinctive ion activation mechanisms on the same precursor demonstrated that HCD was superior to CID in promoting higher sequence coverage and analytical sensitivity in sequence elucidation of T-rich DNA oligonucleotides. Specifically, HCD provided more sequence-defining fragments with higher fragment intensities than CID. Furthermore, the direct comparison between unmodified and PS-modified DNA oligonucleotides demonstrated a loss of MS/MS fragmentation efficiency by PS modification in both CID and HCD approaches, and a resultant reduction in sequence coverage. The deficiency in PS DNA sequence coverage observed with single collision energy HCD, however, was partially recovered by applying HCD with multiple collision energies. Collectively, this work demonstrated that HCD is advantageous to MS/MS sequencing of T-rich PS-modified DNA oligonucleotides.

KEYWORDS
collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), sequencing, synthetic oligonucleotides, tandem mass spectrometry
INTRODUCTION

Oligonucleotide therapeutics have a broad spectrum of indications and comprise a rapidly expanding category of drugs that may change the standard of care for many diseases.1–4 One of the big challenges that therapeutic oligonucleotides face is their instability, since they are highly susceptible to degradation by nucleases in vivo. Various chemical modifications on the base, sugar, or phosphate backbone of synthetic oligonucleotides have been developed to improve their stability to nuclease digestion as well as efficacy and safety.1,3

Structural characterization of synthetic oligonucleotides, including any incorporated modifications, is essential for drug development, manufacturing processes, and quality control of final products. Tandem mass spectrometry (MS/MS) has been shown to provide rapid and reliable structural characterization and sequence determination of synthetic oligonucleotides.5–10 The mass spectrometry-based technique has particular advantages in sequencing modified oligonucleotides, for which the classical enzyme-based methods have faced great challenges due to the presence of modified structural elements. Tandem mass spectrometric sequencing of oligonucleotides, however, is affected by multiple factors, including intrinsic properties of oligonucleotides (e.g., nucleotide composition, sequence, and structural modifications) and experimental parameters (e.g., precursor ion polarity, charge state, and ion activation method).10–14 Thymidine (T)-rich oligonucleotides are notoriously difficult to sequence because of unfavorable bond cleavages at thymidine residues by MS/MS.5,14,15 In addition, structural modifications of oligonucleotides, such as internucleotide phosphodiester linkages replaced by phosphorothioate (PS) linkages, may pose potential challenges to the sequence elucidation by MS/MS because of the added resistance to bond cleavages by the modified linkages.7,8,13,14,16

In this study, sequence characterization of custom synthesized DNA oligonucleotides of the same nucleotide sequence as nusinersen (an 18-mer with seven T units, marketed as Spinraza™ and approved by FDA in 2016) with or without PS modification was investigated using two ion activation techniques: resonant or trap-type collision-induced dissociation (“CID”) and beam-type CID or higher-energy collisional dissociation (“HCD”).15,17,18 The feasibility and suitability of MS/MS fragmentation by CID or HCD were compared for the ability to produce diagnostic fragment ions for the sequence characterization of T-rich PS-modified oligonucleotides.

METHODS

2.1 Materials

DNA oligonucleotides with unmodified (phosphodiester) or modified (phosphorothioate, PS) backbones in sequence TCACCTTCAATAATGCTGG (Mw 5,465.6 and 5,738.6, respectively) were custom synthesized by Invitrogen (Carlsbad, CA). Four more PS-modified DNA oligonucleotides with one nucleotide being deleted from or added to the above sequence were also custom synthesized by Invitrogen, including 17-mer TCCTTTCAATAATGCTGG (Mw 5,409.3), 17-mer TCACCTTCAATAATGCTGG (Mw 5,409.3), 19-mer TCACCTTCAATAATGCTGG (Mw 6,083.9), and 19-mer TCACCTTCAATAATGCTGG (Mw 6,083.9). LC-MS grade ammonium acetate (NH4Ac) was purchased from Sigma-Aldrich (St. Louis, MO). LC-MS grade acetonitrile (ACN) and acetic acid and molecular biology grade HyClone water were from Fisher Scientific (Waltham, MA). LC-MS grade water was from Honeywell (Charlotte, NC).

2.2 Sample preparation

Stock solutions of oligonucleotides were prepared in HyClone water (sterile, for critical applications involving RNA and DNA) and stored at −20°C. The stock solutions were diluted into the appropriate mobile phase to make working solutions at 2 pmol/μl prior to injection.

2.3 LC-MS and MS/MS analysis

LC-MS was performed using Orbitrap Eclipse Tribrid mass spectrometer (Thermo Fisher Scientific) interfaced with a heated electrospray ionization (H-ESI) source (Thermo Fisher Scientific) coupled to a Vanquish Horizon UHPLC system (Thermo Fisher Scientific). Chromatographic separation was performed using a HILIC column (Shodex HiLCpack VN-50 2D, 2.0 × 150 mm, 5 um, 100 Å). The mobile phase additive concentration (15 to 30 mM NH4Ac), mobile phase pH (4.5 to 6.0), column temperature (35°C to 60°C), and MS ion source conditions including spray voltage (2.6 to 3.2 kV), capillary ion temperature (275°C to 400°C), vaporizer temperature (260°C to 350°C), and gas flow rate (sheath and auxiliary gasses, 5.5 to 80) were investigated for their effects on oligonucleotide separation and ionization. The optimized conditions used in this study include the gradient elution of mobile phase A (70% water/30% ACN, containing 20 mM NH4Ac) and mobile phase B (30% water/70% ACN, containing 20 mM NH4Ac). The pH of the NH4Ac solution was adjusted to 5.5 with acetic acid before mixing with ACN. The gradient was 0–1 min 30% A, 1–10 min 30–50% A, and 10–12 min 50% A for unmodified DNA and 0–1 min 40% A, 1–10 min 40–60% A, and 10–12 min 60% A for PS DNA. The flow rate was set at 0.2 ml/min. The column temperature was at 55°C. The sample injection volume was 1.0 μl (i.e., column load of 2.0 pmol of oligonucleotides).

Eluting oligonucleotide anions were analyzed by ESI in negative ion mode. MS scans were performed at a resolution of 120,000 (200 m/z), mass range 600–2,000 m/z, automatic gain control (AGC) target at Standard, and maximum injection time (IT) mode at Auto. Other instrumental parameters include the following: ion funnel radiofrequency (RF) level at 60%; sheath gas, auxiliary gas, and sweep gas flow rates at 55, 5.5, and 0 arbitrary units, respectively; ion transfer tube temperature of 350°C; vaporizer temperature of 300°C; and spray voltage of 3.0 kV. Tandem MS scans (MS/MS) were performed at a resolution of 30,000 (200 m/z), AGC target at Standard, maximum IT mode at Auto, RF level at 30%, and quadrupole isolation width at 0.7 m/z. The collision energy (CE) and AGC target for collision-induced dissociation (CID) were set at 50 and 2.0 × 106, respectively. The LC-MS and MS/MS analysis were performed using Xcalibur 3.0 (Thermo) software.
2 m/z. The most abundant charge state was selected as the precursor ion. The dual fragmentation capabilities of the instrument allow both CID and HCD to be conducted in two separate events in a single experiment. CID or HCD fragmentation was performed at seven different normalized collision energy (NCE) settings ranging from 10% to 40% with an interval of 5% to investigate the impact of NCE on MS/MS fragmentation.

2.4 Data processing

Accurate m/z values of each individual precursor (unmodified or PS modified DNA) and the theoretical fragment ions from predictable backbone cleavages were computed using Agilent Oligonucleotide Sequencer (AOS) 1.4 software. The theoretical fragment ion database for each precursor was composed of the calculated m/z values of all the fragment ions including complementary ion pairs of a-b/W-ions, b/x-ions, c/y-ions, and d/z-ions at varied charge states. MS/MS spectral data were exported to Microsoft Excel using the Qual browser of Xcalibur 4.3. The spectral data were searched against the fragment ion database under a user-defined mass tolerance to identify the fragment ions present and their signal intensities. High resolution and high mass accuracy data enable smaller mass tolerance setting (e.g., ±15 ppm in this study) and thereby more confidence in the peak assignments and the overall accuracy of the sequence confirmation.5,19 A custom Macro program written in Microsoft Excel Visual Basic for Application (VBA) was developed to automate this task. Although commercial software, such as Biopharma Finder 4.0 or above (Thermo Fisher Scientific), may serve as an option for this task, an in-house Macro was used in this study to extract the exact fragment ion intensities for the specific experimental goal of evaluating the analytical sensitivity (see Section 3). The identified fragment ions were used for building a sequence ladder of the sequential fragments and constructing the oligonucleotide sequence map.

3 RESULTS AND DISCUSSION

3.1 HILIC-MS of DNA

For this work, hydrophilic interaction liquid chromatography (HILIC), which uses MS compatible mobile phases, was used as an alternative to more widely used ion-pair reversed-phase liquid chromatography (IPRP LC) to separate oligonucleotides prior to MS analysis.5,7,11,20,22 The absence of ion-pairing (IP) reagents during HILIC-MS analysis helps address some of the IP reagents-related technical hurdles, including (1) ion suppression caused by IP reagents and subsequent reduction in MS signals; (2) instrument contamination by IP reagents, particularly when a dedicated LC-MS system for oligonucleotide analysis is not feasible; and (3) the challenge in selecting appropriate IP reagents and associated acidic modifiers that may be oligonucleotide-dependent. In addition, the relatively higher content of volatile organic solvent at elution of polar analytes by HILIC often provides better ionization and concomitant higher MS sensitivity in comparison to RP LC.

A recently published oligonucleotide HILIC method20 was modified by investigating the impact on oligonucleotide analysis of mobile phase additive concentration and pH, column temperature, and MS ion source conditions. Conditions for achieving a good balance between chromatographic performance (e.g., reasonable retention and good peak shapes) and MS responses (e.g., high ion intensities and proper charge state distributions) were optimized and applied to the HILIC-MS analysis of the custom synthesized DNA oligonucleotide molecules in this study (see Section 2).

The results demonstrated a good peak shape and an envelope of charge state distribution centered on the –4 charge state (Figure 1 inset), which was different from the wide charge state distributions commonly observed during IPRP LC-MS analysis of oligonucleotides.20 Importantly, a predominant charge state offers better analytical sensitivity by consolidating the molecular ion count and is beneficial when using an extracted ion chromatogram for quantitation or when selecting a precursor ion in MS/MS. However, the presence of a single predominant charge state may also lead to the potential loss of analytical flexibility. For example, high molecular weight molecules (e.g., >8,000 Da) may be at risk of being excluded by the available mass range (e.g., up to m/z 2,000) if the major charge state is small (e.g., –4). In addition, if the major charge state cannot be sufficiently broken down to informative fragment ions, a limited sequence coverage may result.13 The former disadvantage was not applicable in this study because the test molecules were 18-mer unmodified or PS-modified DNA oligonucleotides with molecular weights <6,000 Da. Based on an available mass range up to m/z 2,000, the –4 charge state predominance would not be a limiter for therapeutic synthetic oligonucleotides less than 25 nucleotides in length (i.e., <25-mer per single strand), which make up the majority of the oligonucleotide therapeutics currently available. The latter potential disadvantage related to the MS/MS fragment ion analysis is addressed below.

3.2 MS/MS of DNA (unmodified): CID versus HCD

Sequence characterization of oligonucleotides by MS/MS fragmentation of multiply charged oligonucleotide precursor ions in negative ion mode has been intensively studied.5,7 Four possible cleavage sites along the phosphodiester backbone through corresponding dissociation channels can produce four series of sequence-defining complementary ion pairs. As proposed by McLuckey et al.23 in their seminal studies, these four series are designated as a-b/W-ions, b/x-ions, c/y-ions, and d/z-ions, analogous to the nomenclature used for peptides. Many factors have impacts on the MS/MS fragmentation pathways and efficiency, among which the selected precursor ion (e.g., the ion polarity and charge state) and the ion activation method along with associated dissociation conditions are most critical. Since the HILIC-MS under the selected conditions produced one predominant charge state (–4), this charge state was the choice of the precursor to enable
sensitive MS/MS analysis. CID (trap-type dissociation) is conventional resonance excitation where the production of the first-generation fragment ions by single collision is favored. In contrast, HCD (beam-type dissociation) favors a multi-collision dissociation channel and consecutive fragmentation pathway, leading to a greater variety of fragments. In this study, the most abundant [M-4H]4− DNA precursor ion (m/z 1,364.97, Figure 1 inset) was subjected to MS/MS analysis by CID or HCD under varied NCEs to evaluate its fragmentation efficiency and the resultant sequence-defining fragment ions for sequence characterization.

The [M-4H]4− anion did not fragment by CID until the NCE reached 25% (Figure 1A). The nucleobase loss and subsequent 3′ C−O bond cleavage of the abasic deoxyribose led to the most dominant a-B/w-ions as expected for DNA oligonucleotides (Figure 2A,B). In contrast, the [M-4H]4− precursor fragmented by HCD at NCE as low as 10% (Figure 1B). At higher NCEs (e.g., 20% or above), a wide variety of fragment ion types from alternative bond dissociation pathways were observed (e.g., additional b/x and c/y ions produced by phosphodiester P−O bond cleavages) (Figure 2C,D), which shows the dramatic difference in fragmentation behavior from
CID where the fragments were limited to $\alpha$-$B/w$-ions. In addition, the appearance of $\alpha$-type ions that were formed without an initial base loss indicated the presence of a higher energy-promoted dissociation pathway. Those additional ions, however, were mostly produced from backbone cleavages close to the 5' or 3'-ends (e.g., within three nucleotide units) (Figure 1B) and therefore served as confirmatory rather than additional sequence-diagnostic fragments. The higher NCEs also led to the diminishing of diagnostic $\alpha$-$B/w$-ions due to the extensive sequential fragmentation.

Although the quantity and types of fragment ions are critical for assuring a high sequence coverage, the fragment signal intensities are equally critical in rendering MS/MS spectra high-quality (i.e., adequate signal to noise ratio and number of sequence-diagnostic fragment ions), which leads to improved confidence in sequence elucidation that typically becomes more challenging when fragment signal intensity drops. This is particularly important when the precursor ions subjected to MS/MS are in low abundance, such as when sequencing the product-related impurities in synthetic oligonucleotide drugs. Collectively, the NCEs that provided the highest fragment intensities and the most diagnostic fragment ions were 30% for CID and 15% for HCD. Under the optimal NCEs, HCD provided higher total fragment intensities than CID (64% more intense for $\alpha$-$B$ ions and 85% for $w$ ions) at the cost of slightly longer acquisition time required by HCD than CID, in spite of the similar total number of fragments (11 vs. 11 for $\alpha$-$B$ ions and 12 vs. 9 for $w$ ions in HCD vs. CID) (Figure 2).

The sequence coverage was also compared between CID and HCD at the best NCEs (Figure 3). Under both collision types, loss of base T was unfavored, leading to the absence of all six $\alpha$-$B(T)$ ions. Loss of base T is known to be disfavored, particularly for precursors of relatively low charge states. The disfavored T loss is likely attributed to the lower proton affinity of T compared with other nucleobases, which limits the intramolecular proton transfer from the phosphodiester linkage to T that precedes the base loss. However, not all the $w$ ions complementary to $\alpha$-$B(T)$ were missing. Three $w$ ions ($w_{13}$, $w_{5}$, and $w_{2}$) versus one ($w_{13}$ only) were observed despite the absence of corresponding $\alpha$-$B(T)$ ions under HCD versus CID, indicating the presence of alternative fragmentation pathways other than the sequential backbone cleavage following the base loss. In addition, $w_{1}$ ion was also absent under CID due to the low-mass cutoff in ion trap-type instrument (i.e., the “1/3 rule”) that prohibits the detection of low mass sequence ions with m/z values less than 1/3 of the precursor ion m/z. Taken together, fragmentation by HCD was superior to CID for sequencing DNA oligonucleotides by generating a higher quantity and quality of sequence-defining fragments other than the sequential backbone cleavage following the base loss. Similar observations were also reported for peptide sequencing by HCD versus CID. The results demonstrate that complete sequence coverage was achievable on all the non-T nucleotide units by fragmenting the predominant [M-4H] DNA precursor from HILIC-MS by HCD under the optimized collision energy.

### 3.3 MS/MS of DNAs: Unmodified (phosphodiester) versus modified (phosphorothioate)

The PS modification is widely used in synthetic oligonucleotides, in which the naturally occurring phosphodiester linkage is modified by
replacing one of the nonbridging oxygen atoms with a sulfur atom. This backbone substitution greatly improves the nuclease resistance and binding affinity of oligonucleotides. Such modifications, however, may complicate characterization due to modification-induced changes in fragmentation behavior. In this study, MS/MS data for unmodified phosphodiester and modified PS DNA oligonucleotides of the same sequence were collected and directly compared to demonstrate the difference in their sequence elucidation.

As noted with unmodified DNA, a charge state envelope with −4 predominant over others was observed for PS DNA oligonucleotides.

**FIGURE 3** DNA oligonucleotide sequence coverage map using identified MS/MS fragments. On the top sequence panel, the nucleotides that were confirmed by sequence ladders of the identified fragments are highlighted in yellow. On the bottom sequence map panel, the sequence-defining fragments that were identified from MS/MS by both CID (NCE 30%) and HCD (NCE 15%) are highlighted in green for \( \alpha \)-B ions or in blue for \( \omega \) ions. The \( \omega \) ions identified by HCD only are highlighted in light blue. The expected fragments that were not observed from either MS/MS fragmentation are in purple. The negative numbers in parentheses indicate the charge states of the identified fragment ions. The letter in parentheses indicates the nucleobase that is lost in forming an \( \alpha \)-B ion.

**MS/MS analysis of the PS DNA precursor ion \([M-4H]^{4-}\) \((m/z 1,433.12)\) shared a couple of common features with that of unmodified DNA including (1) NCEs ≥25% initiated fragmentation by CID with predominantly \( \alpha \)-B/\( \omega \)-ions; (2) NCE as low as 10% triggered fragmentation by HCD with predominantly \( \alpha \)-B/\( \omega \)-ions; and (3) NCEs ≥20% produced diverse fragment ion types by HCD resulting from additional preferential dissociation channels other than the base loss channel (Figure 4). The optimal NCEs for the studied PS DNA oligonucleotides were 35% for CID and 10% for HCD, where HCD provided a slightly more fragments (9 vs. 8 for \( \alpha \)-B ions and 7 vs. 5 for \( \omega \) ions in...
HCD vs. CID) but significantly higher fragment intensities than CID, that is, 64% more intense for α-B ions and 225% for w ions.

The fragmentation channel initiated by loss of nucleobase T was prohibited for PS DNA regardless of CID or HCD, similar to that observed with unmodified DNA, leading to an absence of all α-B(T) ions. For unmodified DNA, three complementary w ions under HCD and one under CID were present (Figure 3). In contrast, only one complementary w ion (w13) corresponding to the missing α-B5(T) under HCD was observed, and no complementary ions under CID were observed (Figure 5), indicating that alternative fragmentation pathways were less significant in promoting the cleavage of PS linkages compared to that of unmodified phosphodiester linkages. In addition, other missing α-B ions were also observed in MS/MS of PS DNA from the prohibited loss of nucleobases adjacent to T in the sequence (e.g., α-B15[C] and α-B17[G]; Figure 5), contrasting to unmodified DNA where all the missing α-B ions were α-B(T) ions for which the disfavored loss of nucleobase T was fully responsible. The lack of cleavage between T and C residues or between T and G residues was reported previously for DNA precursors of relatively low charge states (e.g., –3). Collectively, the less efficient MS/MS fragmentation of PS DNA in comparison to that of unmodified DNA (i.e., more α-B ions missing and less complementary w ions present) negatively impacted the sequence coverage of PS DNA. The resultant change in the basicity of the PS linkage relative to phosphodiester that suppresses the necessary proton migration may be attributable to the unfavorable bond cleavages in PS DNA.

Interestingly, the additional fragment ions generated by HCD fragmentation of PS DNA under relatively high NCEs (e.g., 20% and above) were in high abundance (Figure 4C) and covered more nucleotide units (e.g., b_n/x_n, c_n/y_n, and d_n ions where n = 1 to 4 from either 5’- or 3’-end) than those of unmodified DNA (Figure 3C, and n = 1 to 3). Those fragment ions could serve as additional sequence-defining fragments (Figure 5, labeled in red in sequence map panel). The larger number of diagnostic fragments could therefore increase sequence coverage by allowing identification of additional nucleotide units (Figure 5, highlighted in orange in sequence panel), rather than only as confirmatory fragments for unmodified DNA. Collectively, the results demonstrate that fragmentation by CID provided an inadequate number of informative fragment ions due to the resistance of PS linkages to cleavage. This resistance, however, was partially overcome by HCD fragmentation under multiple NCEs, low and moderate, such as 10% and 20% for the 18-mer PS DNA in this study. HCD with multiple NCEs promoted complementary fragmentation pathways and the production of a variety of diagnostic fragment ions. Four additional PS DNA oligonucleotide test molecules (two 17-mers and two 19-mers) were also examined under the identified optimal NCEs. Similar results were observed as demonstrated in Figures S1 and S2, including more intense w ions by HCD over CID (Figure S1), missing α-B ions other than α-B(T) ions (i.e., α-B ions highlighted in purple in Figure S2), additional sequence-defining fragments identified under a moderate NCE (i.e., b_n/x_n, c_n/y_n, and d_n ions highlighted in red in Figure S2), and subsequent sequence coverage increase (i.e., identified additional nucleotide units highlighted in orange in sequence panel in Figure S2).

![FIGURE 5](image-url) PS DNA oligonucleotide sequence coverage map using identified MS/MS fragments. On the top sequence panel, the nucleotides that were confirmed by sequence ladders of the identified fragments are highlighted in yellow or orange (see below). On the bottom sequence map panel, the sequence-defining fragments that were identified from MS/MS by both CID (NCE 35%) and HCD (NCE 10%) are highlighted in green or blue for α-B or w ions, respectively. Those identified by HCD only are highlighted in light green or light blue for α-B ions or w ions, respectively. The corresponding nucleotide units in the sequence panel identified by those fragments are highlighted in yellow. The fragments that were identified from an additional MS/MS by HCD under moderate NCE (20%) are in red. The corresponding nucleotide units in the sequence panel identified by those fragments are highlighted in orange. The fragments in purple and the negative numbers and letters in parentheses indicate the same as Figure 3.
4 | CONCLUSION

In this study, a custom synthesized DNA oligonucleotide (18-mer) similar in length to typical antisense oligonucleotide drugs was selected as a test molecule; the oligonucleotide sequence was T-rich (7 over 18 units) and made with or without a PS-modified backbone. The use of HILIC as an alternative to the more frequently used IPRP LC coupled to MS to overcome some of the IP-related technical hurdles was presented. After separation by HILIC, the resultant predominant charge state (i.e., high-coverage) and sensitive (i.e., intense fragment ions) oligonucleotide sequence characterization.

The direct comparison between MS/MS fragmentation by CID and HCD revealed both common and distinctive features. The optimal NCEs selected for the studied DNA oligonucleotide were 30% for CID and 15% for HCD. Both collision mechanisms primarily produced a-B/w-ions; however, HCD facilitated higher fragment intensities than CID. While both collision mechanisms had reduced sequence coverage by the absence of sequence-defining a-B(T) ions, HCD was favored over CID because more complementary w ions were detected from alternative dissociation pathways and HCD had the capacity to detect low-mass fragments that would have been lost due to the low mass (one-third) cutoff of CID.

The impact of backbone PS modification on MS/MS sequence elucidation was investigated. The optimal NCEs identified for the PS DNA oligonucleotide were 30% for CID and 10% for HCD. Similar to unmodified DNA, HCD was superior to CID for PS DNA, generating higher-intensity sequence-defining fragments. In addition, although the resistance of PS linkages to cleavage led to a reduced sequence coverage, some of the unassigned nucleotide units were identified by HCD under relatively high NCEs that produced additional sequence-defining fragment ions. Thus, the sequence coverage of PS DNA by HCD was improved by using the combination of multiple (i.e., low and moderate) NCEs. Collectively, the data in this work showed that tandem mass spectrometry can be successfully applied to structural characterization of synthetic oligonucleotides including challenging T-rich and PS-modified DNA molecules by optimized fragmentation techniques (HCD preferred over CID) and associated collision energies (multiple over single NCEs).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

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