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Application Note

Deciphering Phosphorothioate Isomerism in Synthetic Oligonucleotides Using the Unique Geometry of the Cyclic IMS P20 Mass Spectrometer

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This is an Application Brief and does not contain a detailed Experimental section.

Abstract

Modification of the natural nucleic acid backbone with a phosphorothioate (PS) linker introduces a chiral center, which can adopt either an *R* or *S* configuration. PS stereochemistry can affect the pharmacodynamic and pharmacokinetic properties of oligonucleotide therapeutics, creating a need for advanced analytical approaches to support characterization and quality control in line with regulatory guidelines.

An innovative approach, exploiting the unique geometry of the Cyclic™ IMS P20 Mass Spectrometer, has been developed for characterizing PS stereochemistry at individual linker sites. The method uses Cyclic ion mobility to separate internal dimer fragment ions, each comprising of two nucleotides joined by one PS linker, which is typically a stereo-random mixture of *R* and *S* diastereomers. Using an antisense oligonucleotide (ASO) standard, *R* and *S* diastereomers were separated, allowing quantification of the relative *R*:*S* ratio at the individual linker

level. This approach could be extended to characterization of individual linkers in siRNA and sgRNA, as well as fully phosphorothioated therapeutic ASOs.

Benefits

- Track and control phosphorothioate isomerism in oligonucleotide products with high resolution Cyclic IMS
- Reveal and quantify unresolved isomer ratios through the unique geometry of the Cyclic IMS P20 Mass Spectrometer
- Support evolving regulatory expectations with advanced, high-resolution measurement technologies

Introduction

Oligonucleotide therapeutics have emerged as a novel class of medicines capable of treating a variety of diseases including spinal muscular atrophy, Duchenne muscular dystrophy, hereditary transthyretin amyloidosis, and rare metabolic disorders. Development of chemical modifications to the natural nucleic acid structure was key to overcoming the initial hurdles of efficacy and *in vivo* stability. One of the most common modifications used is the PS linker, which swaps a non-bridging oxygen for a sulfur atom (Figure 1) to improve the properties of the drug. This introduces a chiral center at the phosphorus atom, with each *n*PS linker having either R_p or S_p configuration, leading to the formation of 2^n diastereomers. *R* and *S* are right- (*rectus*) and left- (*sinister*) handed configurations, respectively, and the p denotes the phosphorus atom. For example, for siRNA type molecules with one, two or three PS linkers, this would mean two, four or eight diastereomers, respectively, and so on. For a fully phosphorothioated 20-mer such as an antisense oligonucleotide, there would be 2^{19} = 524,288 isomers.

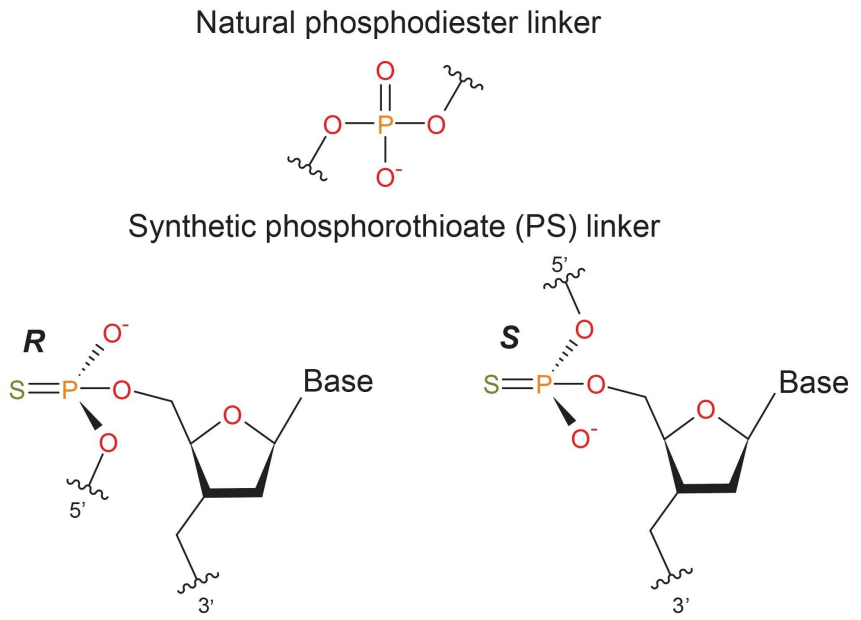


Figure 1. Phosphorothioate linker stereochemistry showing Rp and Sp diastereomers.

The stereochemistry of the PS linkers can affect biological activity, with stereopure versions showing improved biological activity compared to their stereorandom equivalents.[1,2] Aligning with the biological importance, oligonucleotide stereochemistry is under increased scrutiny from regulatory authorities meaning a control strategy is increasingly likely to be required for late phase clinical studies and biosimilars.[3,4]

Current approaches include chromatographic separation with ion-pair reversed phase,[5] HILIC[6] or anion exchange,[7] and NMR has also been shown to enable characterization.[8] Limitations to these approaches include dependence on base sequence, and number and position of PS linkers.

Recent publications have demonstrated that the high-resolution Cyclic ion mobility and the geometry of the Cyclic IMS P20 Mass Spectrometer enables separation and quantification of phosphorothioate diastereomers in therapeutic oligonucleotides from siRNA to sgRNA.[5,9,10-12] In this application brief, the attributes of the Cyclic IMS P20 Mass Spectrometer are highlighted for the determination of stereochemistry ratios at individual PS linkers through the high-resolution ion mobility separation of dimeric internal fragment ions. This novel approach is demonstrated using a 16 nt ASO standard and can easily be extended to therapeutic oligonucleotides.

Results and Discussion

Experimental Strategy

MS/MS is the gold standard for oligonucleotide sequence confirmation by observation of mass ladders built from either end of the oligo, therefore initial investigations into PS chirality naturally focused on these mass ladder fragments. However, terminal fragments such as these can contain multiple linkers, meaning the number of isomers can present a challenge to characterization. The approach in this application brief relies on the generation of internal fragments, formed by cleaving at two sites along the nucleic acid backbone and typically by applying collision energy higher than the optimum required for terminal fragment sequence coverage. The generation of internal fragments presents an opportunity to characterize smaller and more tractable isomer species. Indeed, dimeric fragments, containing only one phosphorothioate, can be observed, providing the opportunity to measure individual PS linker sites.

Figure 2 explains the experimental strategy and shows an example of eight-residue oligonucleotide with two PS linkers, one at linker 2 and one at linker 4. To characterize these linkers, tandem MS is performed on the intact oligo to generate internal fragment ions corresponding to residues 2-3, i.e. dG*dA, and 3-4, i.e. dC*dG. The detected fragment ions are then subjected to multipass ion mobility to separate their *Rp* and *Sp* isomers and determine their ratios. Crucially, this characterization relies on the unique geometry of the Cyclic IMS Instrument, with MS/MS fragmentation being performed in the trap collision cell, situated upstream of the Cyclic ion mobility device.

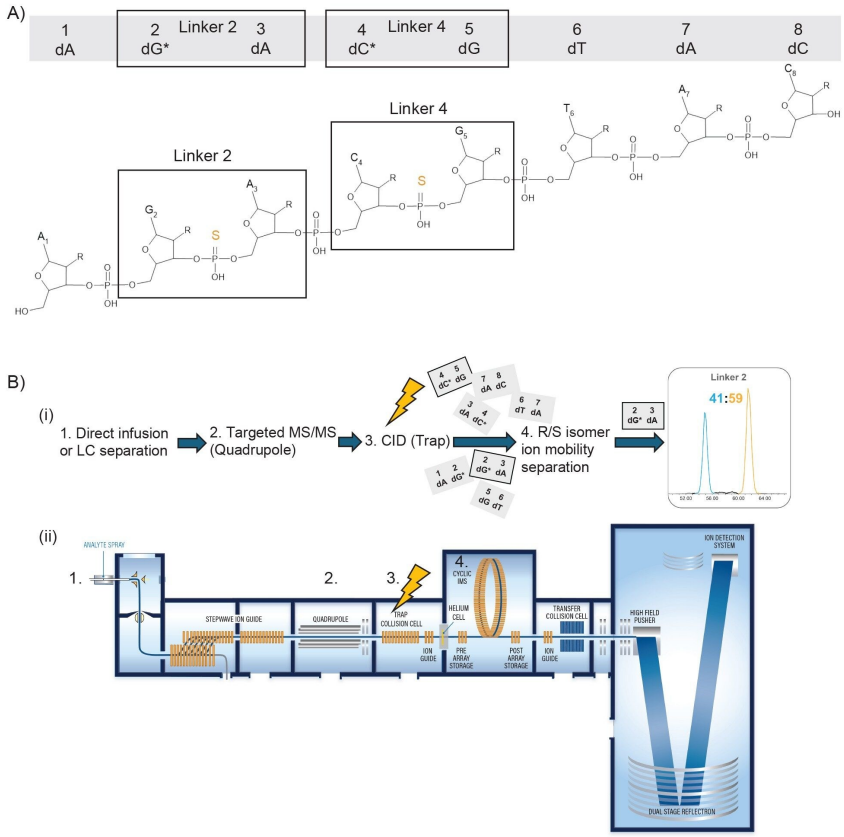


Figure 2. Characteristic fragment ion dimers for PS linker characterization at each linker along a backbone. A) Simple sequence and structural view highlighting nucleotide dimers with PS linkers. B) The experimental approach (i) and the same viewed on the instrument schematic (ii). 1) Samples are introduced into the instrument using either direct infusion or LC separation. 2. A precursor ion charge state is targeted for MS/MS with the quadrupole. 3. CID is performed in the trap. A wide range of fragments are generated, with dimeric fragments being the key ones for this approach. 4. Each dimeric fragment of a PS-linked dimer is subjected to multipass Cyclic ion mobility to separate the R and S isomers from each other. Ratios are reported using peak areas of the arrival time distributions.

Characterizing Individual Linkers with CID-Cyclic IMS

This approach was applied to the Waters Lipid Conjugated Antisense Oligonucleotide (LCASO) LC-MS Standard (Waters, p/n: 186010747 <https://www.waters.com/nextgen/global/shop/standards--reagents/186010747-waters-lipid-conjugated-aso-lc-ms-standard.html>) with representative results shown in Figure 3. Experiments were performed by direct infusion following an offline desalting step, with the possibility to couple the analysis to LC. The full-length oligonucleotide was fragmented in the trap collision cell, and ion mobility settings were optimized to separate the diastereomers of each dimer, enabling characterization of individual PS linkers. Peak areas of the separated diastereomers were determined in MassLynx™ Software and used to determine the relative ratios of *Rp* and *Sp* isomers at each linker. It should be noted that absolute assignment of mobility peaks to the corresponding *Rp* or *Sp* diastereomer would require analysis of stereopure standards or evaluation using computational modelling.

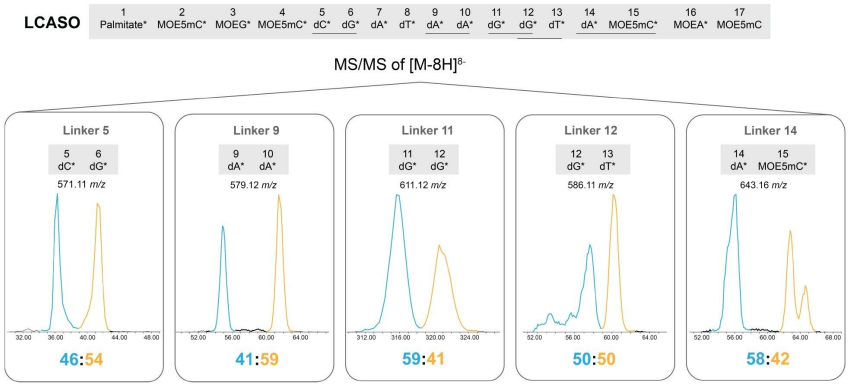


Figure 3. Mobility separated diastereomers for five representative PS linkers of the Waters LCASO LC-MS Standard.

Separating and Assigning Isomeric Dimer Fragments

With only a limited number of residues present in nucleotide products, interrogation of dimeric fragments increases the likelihood of encountering identical or isomeric sequence motifs. For example, a sequence may contain multiple AG as well as GA motifs, each with identical *m/z*.

When this occurs the unique geometry of the Cyclic IMS System with pre-IMS fragmentation is crucial for determining the location of each motif within a sequence. An example from the LCASO is the dimers containing

linker 6 or linker 10; these both contain dG linked to dA, with the *m/z* 595.1242.

There are two approaches for differentiating these isomeric fragment pairs, both of which rely on the generation of larger, ***unique-in-mass*** fragment ions that are further dissociated in pseudo-MS^3 experiments. Fragmentation can be performed either by in-source CID followed by MS/MS or MS/MS followed by IMS^n slicing. Figure 4 shows the two approaches available for uniquely characterizing each individual isomeric linker.

The in-source CID approach utilizes the instrument cone voltage to fragment the oligonucleotide into first-stage product ions, for which the masses can be calculated. Using the quadrupole, these first-stage ions can then be selected based upon their unique mass and further fragmented within the collision cell to break them into second-stage fragments, including dimers. These dimers are then passed into the Cyclic IMS Device for ion mobility separation. Using this approach, the entire charge state distribution of the LCASO was dissociated, followed by mass-selection of first-stage fragment, dG*dA*dT* - which contains only linker 6 and not linker 10 for further fragmentation in the collision cell. It was then possible to separate the *Rp* and *Sp* diastereomers of the dG*dA dimer corresponding to linker 6, determining an isomer ratio of 48:52 ratio (Figure 4, left).

Approach 2 for this isomer pair employed MS/MS of the $[M-8H]^{8-}$ ion using the quadrupole and trap collision cell. In this case, the unique first-generation terminal fragment Y8 was detected and taken forward for investigation. The Y8 ion contained interference, which was removed using mobility selection through IMS^n slicing. Slicing followed by a second round of dissociation generated the dA*dG* dimer corresponding to linker 10, which was then subjected to multipass ion mobility separation and *Rp/Sp* ratio determination.

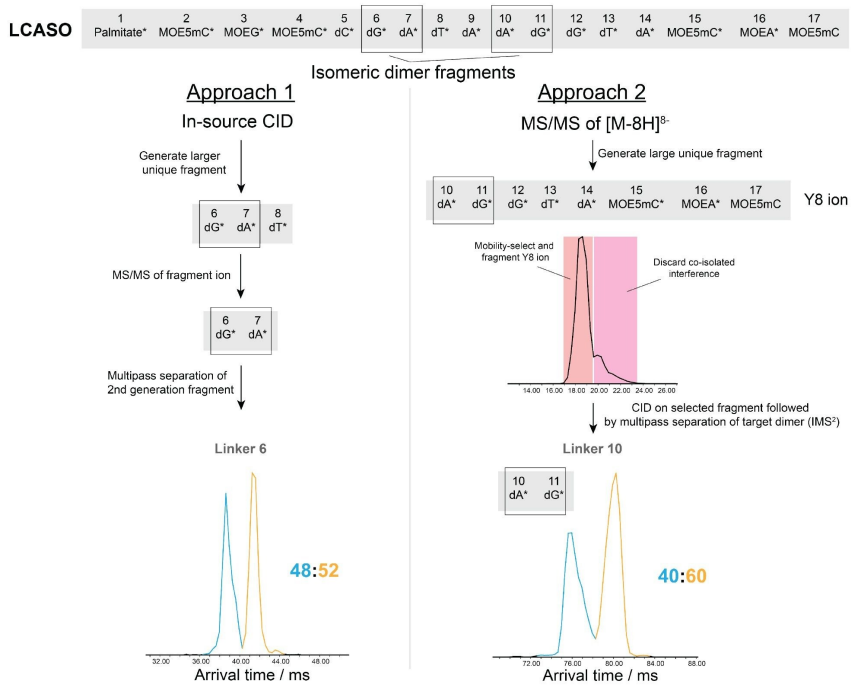


*Figure 4. Approaches for characterization of PS linkers in isomeric dimers utilizing the unique capabilities of the Cyclic IMS P20 System. Approach 1 (left), after electrospray, ions are dissociated in-source using the instrument cone voltage to generate a larger unique fragment. This fragment is then mass-selected using the quadrupole and subjected to a second stage of dissociation to generate the linker 6 dG*dA* dimer. This dimer is then subjected to high-resolution Cyclic ion mobility to determine the isomer ratio of linker 6 (48:52). Approach 2 (right), after electrospray, the precursor ions are quadrupole-isolated and subjected to CID in the trap collision cell to generate a large unique fragment, in this case Y8. Any interference is removed using IMSn slicing and the Y8 ion is dissociated in a second stage of CID upon re-injection into the Cyclic device. The resulting linker 10 dG*dA* dimer is subjected to Cyclic ion mobility separation and isomer ratio determination (40:60).*

Conclusion

Understanding and monitoring PS stereochemistry can provide valuable insight throughout oligonucleotide drug development and commercialization. Characterizing diastereomer composition could help correlate stereochemistry with biological activity, supporting the design of therapeutics with optimized efficacy, improved potency and potentially reduced dosing levels. During process development and scale-up, monitoring stereochemistry can also support process optimization, batch-to-batch consistency and confidence in safe, reliable clinical performance.

These benefits are enabled by the unique geometry of the Cyclic IMS Mass Spectrometer, which allows fragment generation and selection prior to high-resolution, multipass ion mobility separation. The novel approach described here uses this capability to characterize individual PS linker sites through analysis of dimer fragment ions and can be applied across a range of oligonucleotide therapeutics, including antisense oligonucleotides, siRNA and sgRNA.

By combining pre-IMS fragmentation with high-resolution multipass ion mobility separation, the unique Cyclic geometry provides a powerful approach to PS stereochemistry characterization, supporting greater understanding and control of oligonucleotide drug substances and drug products as regulatory expectations evolve.

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