PO-CON1245E

SHIMADZU
Excellence in Science

A Semi-Automated Method for Sequencing Oligonucleotides using ISD and Pseudo-MS^3 on a MALDI-Ion Trap-TOF Mass Spectrometer

IMSC 2012 PWe-147

Matthew E. Openshaw[1], Omar Belgacem[1] and Marco Smith[2]
[1]Shimadzu, Manchester, UK;
[2]GlaxoSmithKline, Stevenage, UK

SHIMADZU
Excellence in Science

A Semi-Automated Method for Sequencing Oligonucleotides using ISD and Pseudo-MS^3 on a MALDI-Ion Trap-TOF Mass Spectrometer

Introduction

Quality Control (QC) analysis of oligonucleotides is commonly performed using MALDI-TOF. Using linear mode analysis, confirmation of the expected mass can be achieved with modest mass accuracy (typically 1-2 Da). An advantage of using MALDI-TOF technology for the analysis of oligonucleotides is the ability to perform in-source decay (ISD) sequencing, if required.

For *unmodified* RNA oligonucleotides, the mass accuracy achieved during ISD analyses is often sufficient to confirm the sequence. However, oligonucleotides designed for use as therapeutics often contain modifications to improve stability and resistance to degradation. In the case of 2' -O-methyl phosphorothioate-modified RNA oligonucleotides, the mass difference between residues can be as small as 1 Da (e.g. modified C = 336 Da, modified U = 335 Da). In such cases, the lower mass accuracy of linear mode ISD may not be sufficient for unambiguous sequence determination.

Here, we describe an approach using ISD performed on a MALDI-Ion Trap-TOF mass spectrometer. The configuration of this instrument is such that high mass accuracy and monoisotopic resolution are achieved for ISD fragments. In a further development of this application, we applied software originally developed for copolymer analysis, for the semi-automated sequencing of the modified oligonucleotides using MALDI-ISD data.

Experimental

Samples were prepared in deionised water and were desalted (x2) using Dowex 50WX8-200 ion-exchange resin beads (Sigma). Samples were prepared using 3-hydroxypicolinic acid (HPA) (Fluka) and ammonium citrate (Fluka). Sample solution/Dowex resin/HPA matrix/ammonium citrate were mixed in a microcentrifuge tube and the resin beads allowed to settle. An aliquot of this solution was deposited onto a stainless steel MALDI target and dried in a vacuum drier box.

Samples were analysed on an AXIMA *Resonance* MALDI-Ion Trap-TOF mass spectrometer (Shimadzu, UK). For ISD experiments, the laser power was increased by approx. 10% compared with that used for MS. Samples were analysed in Mid 850 mode (approx. trapped mass range = ~850 – 3500 *m/z*). 500-800 profiles were acquired (2 shots/profile).
MALDI-ISD data were interpreted in a semi-automated manner using *Polymer Analysis* software (Shimadzu). A tolerance of 200 mDa was used when matching candidate oligonucleotide compositions to the experimental data.

Results

Three 2'-O-methyl phosphorothioate-modifed (2'-OMe) oligonucleotides samples (labelled 1, 2 and 3) were used to test the proposed workflow. The structures of unmodified and 2'-OMe-modified oligonucleotides differ from unmodified oligonucleotides in that: (i) one of the non-bridging oxygens in the backbone phosphate is replaced by sulphur and; (ii) the -OH in the 2' position of the nucleoside is replaced with -OCH_3 .

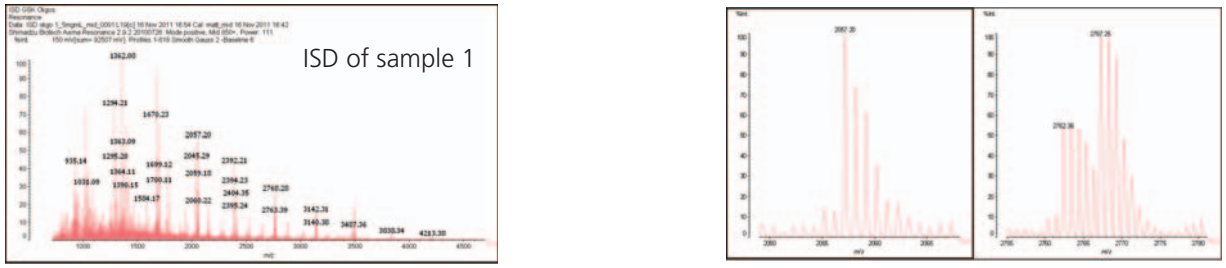


Fig. 1 (*left*) MALDI-Ion Trap-TOF-ISD spectrum obtained for sample 1 (5 mg/mL) and; (*right*) expanded views showing resolution of selected ISD fragments

A Semi-Automated Method for Sequencing Oligonucleotides using ISD and Pseudo-MS^3 on a MALDI-Ion Trap-TOF Mass Spectrometer

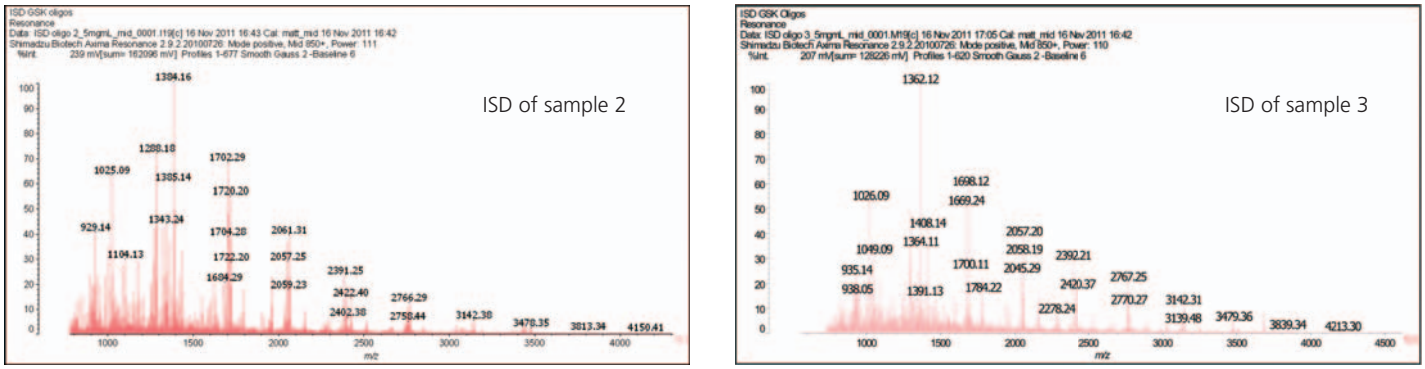
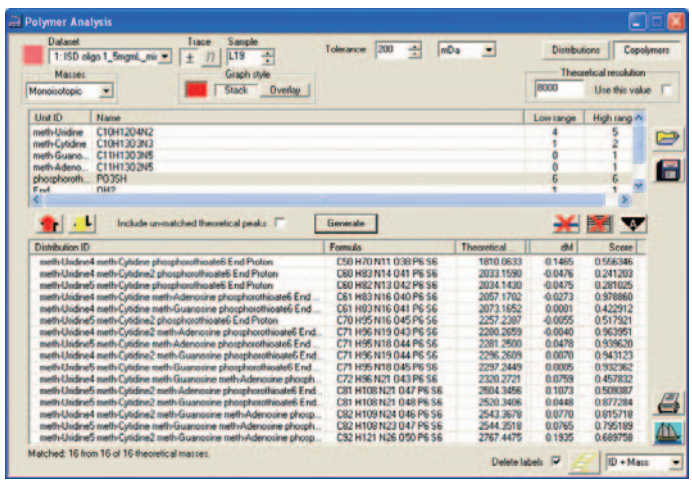


Fig. 2 MALDI-Ion Trap-TOF-ISD spectra obtained for: (*left*) sample 2 (5 mg/mL) and; (*right*) sample 3 (5 mg/mL)

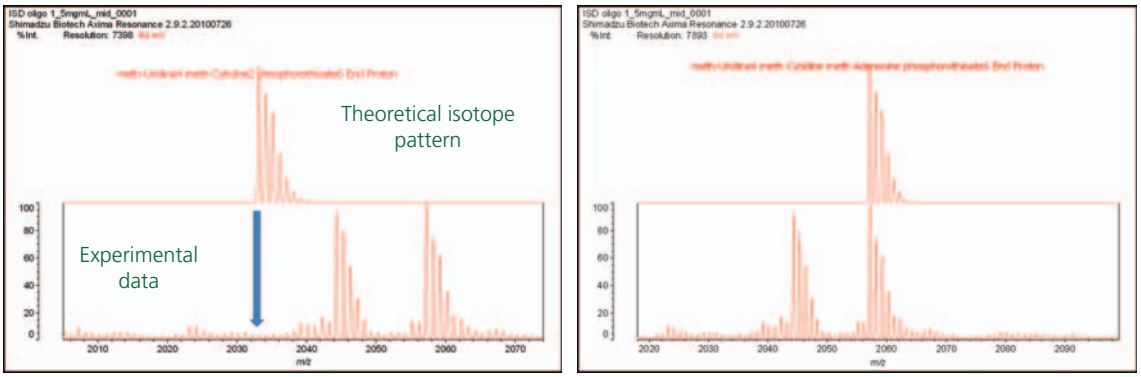


The *Polymer Analysis* software (*left*) was originally developed for the analysis of copolymer samples. The user defines the elemental composition of the individual components and specifies the minimum and maximum permitted number of each to be used in the calculations (see the 'Low range' and 'High range' fields). For example, in the case of the ionising proton, the min. and max. limits were set to 1(i.e. a maximum of 1 proton must be present in each calculated composition). The software then calculates all possible combinations of the various components (within the specified min./max.limits) and compares the results against the experimentally obtained spectrum. If a peak is detected in the mass spectrum, within the user-defined tolerance, the match is listed in the *Polymer Analysis* software and the mass error is shown. In addition, a score is calculated based on the isotopic pattern fit relative to the theoretical pattern (see Fig. 4 below).

Fig. 3 *Polymer Analysis* software screenshot showing the *Copolymers* analysis mode which was used in this work.

As oligonucleotides are composed of only 4 residues, we proposed that the *Polymer Analysis* software may facilitate sequencing of this class of compound. The composition of the 4 residues (A, C, G and U), the adduct (H^+), the modified-phosphorothioate backbone and the end groups were all specified. Sequencing was performed in a stepwise manner: initially, limits for the 4 residues were relaxed (0 (min.) to 4 (max.)). If a single composition was detected for one of the main fragments in the lower *m/z* region (1000-1400 *m/z*), this was taken as the starting composition. [It should be noted that for this first terminal fragment, the composition is proposed but the sequence order is unknown]. Subsequently, the low limits for the various residues were set to match the composition of the proposed fragment while the high limit for all residues was increased by 1. Similarly, the low- and high-limits for the number of phosphorothioate groups was set to n+1, where n = the number of residues from the initial proposed composition. In this way, the sequence of the oligonucleotide could be extended 1 residue at a time. Finally, the sequence order of the proposed 3'- and 5'-terminal fragments was determined using pseudo-MS^3 (see Fig. 5).

A Semi-Automated Method for Sequencing Oligonucleotides using ISD and Pseudo-MS^3 on a MALDI-Ion Trap-TOF Mass Spectrometer



2033.2 *m/z* (score = 0.24)
Poor match

2057.2 *m/z* (score = 0.98)
Good match (correct)

Fig. 4 (right): Polymer Analysis software results obtained during semi-automated oligonucleotide sequencing. The upper trace shows the theoretical isotope pattern for the selected composition and the lower trace shows the experimental data.

In Fig. 3 (Polymer Analysis software screenshot), from the iteration shown, 2 compositions are proposed which are valid based on the ratio of 'number of residues' and 'number of phosphorothioate residues' (# residues = # phos groups). Selecting the proposed compositions allows comparison of the theoretical isotope pattern with the experimental data obtained (see Fig. 4). Using the *Polymer Analysis* score (based on isotopic pattern fit), the incorrect composition (2033.2 *m/z* (Fig. 4 (a))) can be quickly eliminated.

The full oligonucleotide sequence was determined by combining sequence information obtained from both the 5'- and 3'-termini. For sample 2, it was not possible to confirm the central 2 residues (shown as XX). Using the intact molecular weight for the sample (determined by linear mode MALDI-MS, not shown) and the determined 5'- and 3'-sequences, the missing residues were consistent with either CC, [CU] or UU. A higher mass accuracy measurement of the precursor would help eliminate such ambiguities.

For all the samples, the composition of the terminal sequences were proposed in the first iteration but the sequence order was not known. The termini sequences (typically approx. 4 residues) can be confirmed by performing pseudo-MS^3 (i.e. MS/MS of the ISD fragment .See Fig. 5). The oligonucleotide samples used in this work were synthetic products and the theoretical sequence was used to facilitate interpretation of the MS/MS spectra for the terminal residues.

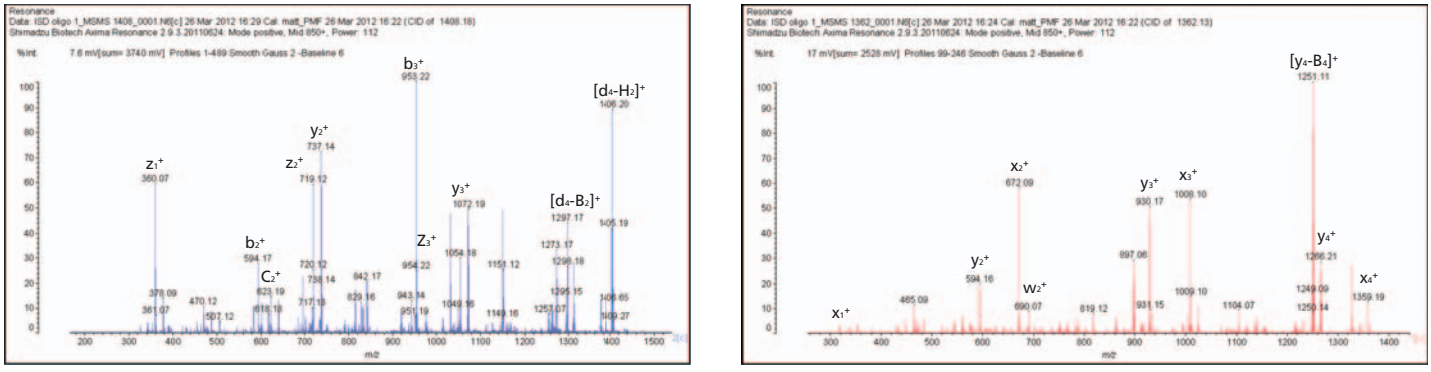


Fig. 5: Pseudo-MS^3 (i.e. MS/MS of ISD fragments) of terminal fragments of sample 1:
(*a*) 5'-terminal fragment (1408.1 *m/z*; [UCAA…]) and;
(*b*) 3'-terminal fragment (1362.1 *m/z*; […UUCU])

A Semi-Automated Method for Sequencing Oligonucleotides using ISD and Pseudo-MS^3 on a MALDI-Ion Trap-TOF Mass Spectrometer

Table 1 shows the sequences determined for samples 1, 2 and 3. The termini sequences (underlined in table 1) were confirmed using the theoretical oligonucleotide sequence and the MS/MS data.

Sample	Sequence obtained	# Correct residues (% correct)
1	UCAAGGAAGAUGGCAUUUCU	20/20 (100 %)
2	GGCCAAACCXXGGCUUUCCA	16/20 (80 %)
3	UCAAGGGAAGAUGGCAUUUCU	21/21 (100 %)

Table 1: Summary of sequencing results obtained for oligonucleotide samples 1, 2 and 3. For sample 2, the residues shown as XX could not be determined as the higher mass ISD fragment ions were too low abundance.

Note: sequence confirmation of terminal residues (underlined) was performed using the theoretical oligonucleotide sequence.

Conclusion

- The advantages of the MALDI-ion trap-TOF compared with a regular linear mode MALDI-TOF for in-source decay include the high mass accuracy and monoisotopic resolution obtained for the ISD fragments and the ability to perform high quality pseudo-MS^3 for terminal sequence confirmation.
- However, the higher mass ISD fragments towards the middle of the sequence have lower abundance which limits the size of the oligonucleotide which can be fully sequenced (approx. 20-mer). Full sequence coverage can be determined by combining sequences from both termini. In the case of sample 2, portions of the sequence were determined incorrectly.
- The *Polymer Analysis* software was shown to be applicable for the semi-automated sequencing of oligonucleotides using MALDI-ion trap-TOF ISD data, although further optimisation is still required. As shown, this software can be used in an iterative approach to determine the sequence of one residue at a time. However, manual verification of the correct residues is still required although this could be automated in the future using simple rules to exclude sequences which are not possible.

First Edition: September, 2012

SHIMADZU

Shimadzu Corporation

www.shimadzu.com/an/