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Development of a quality control method for the characterization of oligonucleotides by capillary liquid chromatography-nano-electrospray ionization quadrupole time-of-flight mass spectrometry.

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1. Introduction

- Synthetic oligonucleotides e.g. PCR primers, probes, antisense therapeutics ⇒ no defect in length or sequence tolerated
- Quality control and characterization of oligo's after synthesis: accurate and rapid structural identification and purity determination
- Analytical tool: electrospray ionization mass spectrometry (ESI-MS)
  - molecular weight determination
  - deconvolution algorithm produces zero charged spectrum from multiply charged ESI raw spectrum
  - single base substitutions? (between 9 and 40 Da)
- Problem: adduction of sodium or potassium ions to polyanionic backbone
  ⇒ highly complex mass spectra
- Desalting possible by replacement of cations with ammonium or trietylammonium ions (less tightly bound to oligo, dissociate during electrospray process) or by abstraction of cations by chelating agents (e.g. *trans*-1,2-diaminocyclohexane-*N,N,N,N*-tetraacetic acid (CDTA))
- Objective: development of an on-line capillary ion-pair reversed-phase liquid chromatograpy (IP-RP-LC)-negative nano-ESI-MS method combined with column switching using a Q-TOF mass analyzer for the characterization of oligo's including concomitant removal of salt ions

2. Experimental

Oligonucleotide samples:

- Samples (Applied Biosystems): Table 1 (between 125 and 180 pmol/µL)
- Oligonucleotide 3: model oligo for the development of the method

LC-MS conditions:

- Autosampler: Famos™; Column switching: Switchos™; Pump: Ultimate™ (LC Packings, The Netherlands)
- Column: microguard column, C18 Pepmap®, 300 µm i.d., 5 µm particle (LC Packings, The Netherlands)
- Mass Spectrometer: Q-TOF hybrid mass spectrometer (Waters, Manchester, UK) equipped with a nano-electrospray source (Z-spray®)
- Capillary: PicoTip™ emitter, coated SilicaTip™ (New Objective, Woburn, MA, USA)
- Negative ESI voltage: -2.5 to -2.8 kV, sample cone: 35-45 V
- After injection (10 µL), the column, used for compound trapping and separation, was loaded using 0.4 M 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in water (adjusted to pH 7.0 with triethylamine (TEA), including 10 mM ammonium acetate, loading solvent) at a flow rate of 12 µl/min. After a preset loading time, a valve switch was initiated and the separation was started using 0.4 M HFIP in 50/50 methanol/water (adjusted to pH 7.0 with TEA, elution solvent) at a flow rate of 0.8 µl/min.

3. Results and discussion

Goal

- Optimization of an on-line capillary LC-ESI-MS method for the desalting and characterization of oligonucleotides in one single step by changing the composition of the diluting solution of the samples and by changing the loading process (composition of loading solvent, loading time)
- Results
  - Bar diagram: sum of the signal abundances (peak heights) of the different multiply charged ions of oligonucleotide 3 and of all the observed adducts, extracted from the full scan spectra of the sample
  - Line diagram: ratio of the sum of the peak heights of the different multiply charged ions of oligonucleotide 3 and the sum of the peak heights of all the observed adducts (the higher the ratio, the better the desalting occurred)

Optimization of the composition of the diluting solution of the samples

- Dilution of oligo sample to 0.36 pmol/µL with 0.4 M HFIP in water (pH 7.0), loading/washing of the microguard column during 4 minutes with 0.4 M HFIP in water (pH 7.0), followed by elution of the oligo with 0.4 M HFIP in 50/50 methanol/water (pH 7.0) into the mass spectrometer
  - retention time 13-14 minutes
  - BUT: still extensive cation adduction, especially potassium adducts (Figures 1 and 2, Figures 3 and 4 (number 1))

  ⇒ further reduction of cation adduction imperative

- Dilution of oligo sample to 0.36 pmol/µL with 0.4 M HFIP in water (pH 7.0) supplemented with 1/2/3, loading/washing of the microguard column during 4 minutes with 0.4 M HFIP in water (pH 7.0), followed by elution of the oligo with 0.4 M HFIP in 50/50 methanol/water (pH 7.0) into the mass spectrometer (Figures 3 and 4)
  1. Varying concentrations of ammonium acetate (0.1, 0.3, 0.5 or 1 M, number 2, 3, 4 and 5 in Figures 3 and 4)
     - replacement of metal ions with ammonium ions
     - partial displacement of sodium and potassium adducts
     - substantial reduction of analyte signal if 0.3 M ammonium acetate or more is used for dilution of the sample
  2. Varying concentrations of CDTA (1, 3, or 5 nmol/ 100 pmol oligo, number 6, 7 and 8 in Figures 3 and 4)
     - abstract metal ions from the oligonucleotide
     - increase of oligonucleotide signal abundance as well as the amount of adducts
  3. 0.5% formic or acetic acid (number 9 and 10 in Figures 3 and 4)
     - charge state reduction
     - decrease of oligonucleotide signal abundance
- Conclusion: dilution of oligo sample with 0.4 M HFIP in water supplemented with 0.1 M ammonium acetate ⇒ best result for reduction of cation adduction

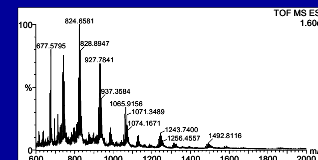


Figure 1: Spectrum of oligonucleotide 3.

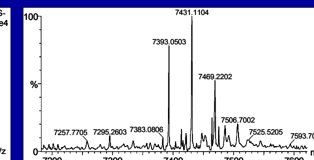
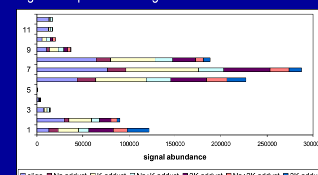


Figure 2: Deconvoluted spectrum of oligonucleotide 3.



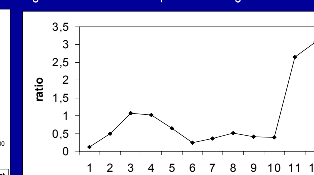
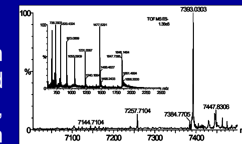
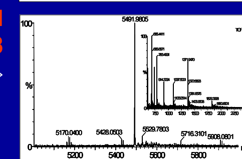


Figure 3 (left): Signal abundance of oligo and adducts. Figure 4 (right): Ratio of signal abundance of oligo and adducts.

Optimization of the loading process

Dilution of oligo sample to 0.36 pmol/µL with 0.4 M HFIP in water (pH 7.0) supplemented with 0.1 M ammonium acetate, loading/washing of the microguard column with 0.4 M HFIP in water (pH 7.0) + 10 mM ammonium acetate during 4 minutes or 8 minutes (number 11 or 12 in Fig. 3 and 4) ⇒ 8 minutes best results (Figure 5)





Figures 5 and 6: Spectrum and deconvoluted spectrum of oligonucleotide 3 (up, Fig. 5) and oligonucleotide 2 (down, Fig. 6).

Applications

- 7 other oligonucleotides (Table 1, average of 3 measurements + standard deviation), little adducts observed in spectra (example Fig. 6)
- Maximum errors < 70 ppm or 0.4 Da ⇒ smallest difference (A to T switch differing 9 Da in mass) can be detected

No	Base composition	Expected MM	Observed MM (SD)	ppm
1	5'-CCA CCA TGC CAC CTC CT-3'	5011.3092	5011.5036 (0.0462)	38.8
2	5'-GGT GCT CCA GGT GCC CAT-3'	5491.6027	5491.9803 (0.0301)	68.8
3	5'-CCC TGG GCT CTG TAA AGA ATA GTG-3'	7392.8587	7393.0038 (0.1125)	19.6
4	5'-ATC AGA GCT TAA ACT GGG AAG CTG-3'	7425.8973	7426.0638 (0.0450)	19.9
5	5'-AAT AAG CTT CCA CCA TGC CAC CTC CT-3'	7795.1307	7795.4372 (0.1513)	39.3
6	5'- ATT GTC GAC GGT GCT CCA GGT GCC CA-3'	7963.2024	7963.4736 (0.0853)	34.1
7	5'- ATT GTC GAC GCT CTT CAT CCT CGT TCT CA-3'	8745.7164	8745.9372 (0.1161)	25.2
8	5'-ATT GTC GAC CAC AGC TGA GAC CTT CCA GCC-3'	9111.9673	9112.3473 (0.0501)	41.7

Tabel 1: Oligonucleotides used in this study.

4. Conclusion

It is concluded that the capillary-LC-nano-ESI-MS method with column switching can remove salt ions, deleterious for mass spectrometric oligonucleotide length and sequence analysis. The procedure, which combines trapping and separation in a single step, is rapid and fully automated, so it is ideally suited for the quality control of oligonucleotides.

5. Acknowledgements

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