Development of a quality control method for the analysis of oligonucleotides by capillary zone electrophoresis-electrospray ionization mass spectrometry

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1. Introduction

Synthetic oligonucleotides e.g. used as PCR primers or antisense therapeutics have to be of high purity and a defect in length or sequence is not tolerated. Electrospray ionization mass spectrometry (ESI-MS) has become a very important analytical tool for the characterization of these oligonucleotides after synthesis, based on a difference in mass. However, the major difficulty is the adduction of sodium or potassium ions to the polyanionic backbone, resulting in highly complex spectra and decreased sensitivity. Removal of these metal ions can be accomplished by the addition of organic bases, e.g. piperidine and imidazole, or the addition of *trans*-1,2-diaminocyclohexane-*N,N,N,N*-tetraacetic acid (CDTA) to the spray solution, the multiple ammonium acetate precipitation method, or on-line microdialysis (1-3). The essence of some of these procedures involves the replacement of sodium ions with ammonium, which has been shown to considerably reduce sodium adduct formation in ESI-MS. Most of the approaches for the reduction of cation adduction are time-consuming, require relatively large amounts of sample or are off-line. In that respect, we developed an on-line capillary zone electrophoresis (CZE)-negative ESI-MS method using a Q-TOF mass analyzer for the analysis of oligonucleotides including concomitant removal of salt ions. The advantage of our method is that little sample is necessary and that the metal ions are exchanged for ammonium ions during separation in the ammonium carbonate buffer, thus eliminating the need for sample preparation steps. Parameters optimized included pH, buffer concentration and composition, sheath liquid composition, applied CZE voltage and negative ESI conditions to obtain stable electrospray conditions, the best signal response for the oligonucleotides and a maximum reduction of the nonvolatile cations while maintaining a reasonable analysis time.

2. Materials & Methods

Oligonucleotides: The oligonucleotide samples (Applied Biosystems) used in this study are displayed in Table 1, together with their expected average and observed molecular mass. The concentration of the oligonucleotides was between 125 and 180 nmol/mL. Oligonucleotide 3 was used as model oligonucleotide for the development of the method. The synthesized oligonucleotides were not purified or desalted before analysis.

CZE-ESI-MS Conditions:

- CZE system: PRINCE (Lauerlabs), fused silica capillary (0.86m x 50µm i.d.)
- Mass spectrometer: Q-TOF (Waters, Manchester, UK)
- Ion source: triaxial nano-electrospray source (Z-spray®) in the negative ion mode
- Hydrodynamic injection (100 mbar, 1 min) followed by preconcentration on the capillary using sample stacking
- Electrophoresis buffer: 25 mM ammonium carbonate (pH 9.7) (+ 2.5 mM piperidine and imidazole/+ 0.2 mM CDTA/+ 2.5 mM piperidine and imidazole + 0.2 mM CDTA)
- Electrophoretic conditions: 14 kV, 60 mbar
- (-)-ESI conditions: capillary voltage –3.0 kV, cone voltage 35 V, source temperature 80°C, desolvation gas flow rate 125 L/h and nebulization gas pressure 1.2 bar.
- Sheath liquid: 80/15/5 isopropanol/water/0-20 mM ammmonium carbonate pH 9.7 (0.7 µl/min).

3. Results and discussion

In preliminary experiments, the electrophoresis voltage and hydrodynamic pressure were selected using a 25 mM ammonium carbonate buffer. A very stable electrospray and a reasonable analysis time of about 25 min were achieved when electrophoresis was performed using a constant voltage of 14 kV during which a constant pressure of 60 mbar was applied. The pH was optimized at pH 9.7. Using these conditions, there were still sodium and potassium adducts visible in the raw electrospray negative ion spectrum as well as in the deconvoluted spectrum (Figure 1).

In an attempt to improve the sensitivity through obtaining further reduction of the cation adduction, a 50 mM ammonium carbonate solution (migration time 28 min); and the addition of 2.5 mM piperidine and imidazole (Figure 2), the addition of 0.2 mM CDTA (Figure 3) and the addition of 2.5 mM piperidine and imidazole + 0.2 mM CDTA to the initial buffer system were tested. The results are compiled in Figure 4. They are expressed as the ratio of the sum of the peak heights of the different multiply charged ions of the oligonucleotide and the sum of the peak heights of all the observed adducts (left axis) and as the signal intensity of oligonucleotide 3, which is the sum of the peak heights of the different multiply charged ions of the oligonucleotide (right axis), extracted from the full scan spectrum of the sample. The signal intensities are means (n = 2). The highest increase of the signal response of the oligonucleotide together with a marked reduction of cation adducts is accomplished using 25 mM ammonium carbonate + 0.2 mM CDTA (pH 9.7) as electrophoresis buffer.

No.	Base composition	Expected average MM	Observed MM	Error (ppm)
1	5'-CCA CCA TGC CAC CTC CT-3'	5011.3092	5011.1401	-33.7
2	5'-GGT GCT CCA GGT GCC CAT-3'	5491.6027	5491.4902	-20.5
3	5'-CCC TGG GCT CTG TAA AGA ATA GTG-3'	7392.8587	7392.8901	4.2
4	5'-ATC AGA GCT TAA ACT GGG AAG CTG-3'	7425.8973	7425.6704	-30.6
5	5'-AAT AAG CTT CCA CCA TGC CAC CTC CT-3'	7795.1307	7795.1006	-3.9
6	5'-ATT GTC GAC GGT GCT CCA GGT GCC CA-3'	7963.2024	7963.2803	9.8
7	5'-ATT GTC GAC GCT CTT CAT CCT CGT TCT CA-3'	8745.7164	8745.7402	2.7
8	5'-ATT GTC GAC CAC AGC TGA GAC CTT CCA GCC-3'	9111.9673	9111.9004	-7.3

Table 1: Oligonucleotides used in this study.

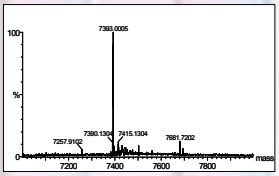

Figure 3: Deconvoluted spectrum after addition of 0.2 mM CDTA to the running buffer.

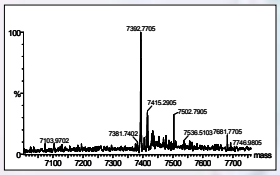

Figure 1: Deconvoluted spectrum using 25 mM ammonium carbonate (pH 9.7) as running buffer.

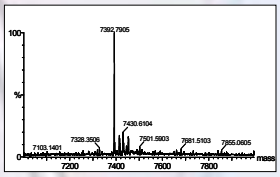

Figure 2: Deconvoluted spectrum after addition of 2.5 mM piperidine and imidazole to the running buffer.

In a next step, the addition of 5% 2-20 mM ammonium carbonate buffer (pH 9.7) to the sheath liquid was investigated for further improvement of the signal. Addition of 5% 5 mM ammonium carbonate buffer (pH 9.7) to the sheath liquid gave the best improvement of the signal and the most stable electrospray, together with the lowest amount of adducts (Figure 5). Finally, the applied voltage was tested in the range from 12 to 17 kV to obtain a maximum signal response of the oligonucleotide together with a minimum signal response of cation adducts. This result was achieved applying a voltage of 14 kV during electrophoresis (Figures 6 and 7).

The optimized method was tested with 7 other oligonucleotides, which are listed in Table 1. Figure 8 shows the deconvoluted spectrum of oligonucleotide sample 6. There were no sodium or potassium adducts observed in this spectrum. As can be seen from Table 1, the maximum errors obtained on the mass determination of these short oligonucleotides are less than 35 ppm. For the mass range used in this study, this corresponds to an error of about 0.3 Da. It is obvious from these data that even the smallest difference (A to T switch differing 9 Da in mass) can be detected without any problem.

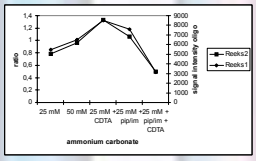

Figure 4: Effect of concentration and composition of the buffer on the signal intensity of oligo 3 and its adducts (n=2).

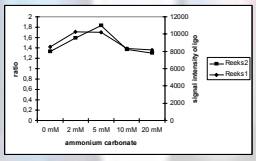

Figure 5: Effect of addition of 5% 0 to 20 mM ammonium carbonate to the sheath liquid on signal intensity of oligo 3 and its adducts (n=3).

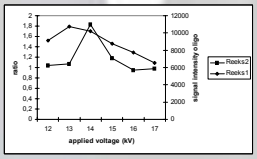

Figure 6: Effect of applied voltage on signal intensity of oligo 3 and its adducts (n=2).

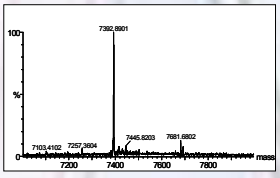

Figure 7: Deconvoluted spectrum after addition of CDTA to the running buffer and addition of 5% 5 mM buffer to the sheath liquid, applied voltage 14 kV.

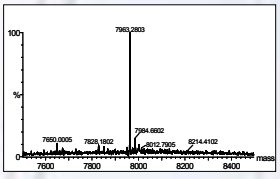

Figure 8: Deconvoluted spectrum of oligonucleotide sample 6.

4. Conclusion

It is concluded that the CZE-ESI-MS method with on-line sample stacking can remove salt ions of oligonucleotide samples, deleterious for mass spectrometric oligonucleotide length and sequence analysis. The procedure uses minimal labor and little sample, thus is ideally suited for the quality control of oligonucleotides.

5. Acknowledgements and references

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