Journal of Chromatography A, 1218 (2011) 5480–5486

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Analysis of oligonucleotides by hydrophilic interaction liquid chromatography coupled to negative ion electrospray ionization mass spectrometry

Lingzhi Gong, James S.O. McCullagh *

Chemistry Research Laboratory, Department of Chemistry, University of Oxford, Oxford OX1 3TA, United Kingdom

ARTICLE INFO

Article history:
Received 18 January 2011
Received in revised form 31 May 2011
Accepted 9 June 2011
Available online 17 June 2011

Keywords:
LC/MS
Oligonucleotides
HILIC chromatography
MS friendly buffer

ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) is here successfully coupled to negative-ion electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) for the analysis of synthetic and chemically modified oligonucleotides. Separation was performed on a 2.1 mm × 100 mm PEEK ZIC® HILIC column packed with hydrophilic stationary phase with a permanent zwitterionic functional group and a particle size of 3.5 μm with an average pore diameter of 200 Å. A method was developed to separate homogeneous and heterogeneous oligonucleotides as well as methylated oligonucleotides using a quaternary pumping system containing ammonium acetate and water with an acetonitrile gradient. Analyses of oligonucleotides were performed by LC/MS with a detection limit of 2.5 picomole (20 mer) with signal to noise ratio (S/N) of 4.12. The influence of the eluent composition, type of buffer and its concentration, and organic modifier were also evaluated. The HILIC LC/MS method presented in this paper used common, 'MS friendly', mobile phases achieving sensitive and selective oligonucleotide analysis.

1. Introduction

Sensitive methods for oligonucleotide separation, identification and purification have become increasingly important for research in chemical and molecular biology. Unmodified oligonucleotides are widely used in the polymerase chain reaction (PCR) as DNA primers or as probes for detecting DNA and RNA [1–3]. Modified oligonucleotides are used as therapeutics [4,5]. To purify and analyze oligonucleotides, fast and robust methods are required with high sensitivity and selectivity, especially, when the oligonucleotides are applied as therapeutics [6–8]. Chromatographic techniques such as high-performance liquid chromatography (HPLC) [9–13] and electrophoresis [14–17] have for a long time been used in the purification and analysis of oligonucleotides.

Both matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [18–20] and electrospray ionization mass spectrometry (ESI-MS) [21–23] have been used in the sequencing, identification, and characterization of oligonucleotides, as well as for genotyping applications [24,25]. However, the detection and characterization of oligonucleotides by ESI-MS results in complex mass spectra with a tendency towards low signal to noise ratios, and poor mass accuracy. This can often be due to the polyanionic nature of oligonucleotides leading to the formation of quite stable heterogeneous adducts with cations, such as sodium, potassium and iron ions, that may be present in the sample or mobile phase. Hence, sample pre-treatment by removing adducts off-line [26,27] or on-line [28–30] is essential in order to obtain high-quality mass spectra. HPLC has been widely used as an on-line sample preparation technique [31,32] and it has the advantage of simple coupling to mass spectrometry (LC/MS) combining desalting and separation with identification. Ion-pair reversed-phase liquid chromatography (IP-RP HPLC) has been widely used for analysis of oligonucleotides [33–37] since Apffel et al. [38] introduced an ion-pairing and buffered system comprised of triethylamine (TEA) as the ion-pairing agent and hexafluoroisopropanol (HFIP). Although these ion-pairing systems improved LC/MS performance, there was a compromise between chromatographic resolution and MS sensitivity.

An alternative chromatographic approach to the separation of polar and hydrophilic substances is hydrophilic interaction liquid chromatography (HILIC), a version of normal phase liquid chromatography. The name was suggested by Alpert [39] in his 1990 paper on the subject who proposed that the HILIC mechanism involves the mobile phase forming a water-rich layer on the surface of the polar stationary phase which interfaces with the water-deficient mobile phase, creating a liquid/liquid extraction system. Polar analytes are retained and separated primarily by partitioning between these two layers. The high organic content of the mobile phase makes HILIC particularly compatible with electrospray ionization but it is important that the analyte solvent is also high in organic content, ideally close to the mobile phase starting conditions. Needless to say many biomolecules are

* Corresponding author.
E-mail address: james.mccullagh@chem.ox.ac.uk (J.S.O. McCullagh).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.06.044

Table 1
Sequences and molecular properties of oligonucleotides used in this study.

Name	Sequence	Length (mer)	M_r (Da)
dT15	TTT TTT TTT TTT TTT	15	4501.0
dT16	TTT TTT TTT TTT TTT T	16	4805.2
dT17	TTT TTT TTT TTT TTT TT	17	5109.4
dT18	TTT TTT TTT TTT TTT TTT	18	5413.6
dT19	TTT TTT TTT TTT TTT TTT T	19	5717.8
dT20	TTT TTT TTT TTT TTT TTT TT	20	6022.0
dT21	TTT TTT TTT TTT TTT TTT TTT	21	6326.2
dT22	TTT TTT TTT TTT TTT TTT TTT T	22	6630.3
dT23	TTT TTT TTT TTT TTT TTT TTT TT	23	6934.5
dT24	TTT TTT TTT TTT TTT TTT TTT TTT	24	7238.7
dT25	TTT TTT TTT TTT TTT TTT TTT TTT T	25	7542.9
dT26	TTT TTT TTT TTT TTT TTT TTT TTT TT	26	7847.1
dT27	TTT TTT TTT TTT TTT TTT TTT TTT TTT	27	8151.3
dT28	TTT TTT TTT TTT TTT TTT TTT TTT TTT T	28	8455.5
dT29	TTT TTT TTT TTT TTT TTT TTT TTT TTT TT	29	8759.7
dT30	TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT	30	9063.9
dA20	AAA AAA AAA AAA AAA AAA AA	20	6202.2
dC20	CCC CCC CCC CCC CCC CCC CC	20	5721.6
EVEN	GGG GGC CCC CAA AAA TTT TT	20	6117.0
NEW EVEN	GTC AGT CAG TCA GTC AGT CA	20	6117.0
EVEN G	GGG GGC CCC CAA AAA TTT TTG	21	6446.2
EVEN C	GGG GGC CCC CAA AAA TTT TTC	21	6406.2
EVEN A	GGG GGC CCC CAA AAA TTT TTA	21	6430.2
EVEN T	GGG GGC CCC CAA AAA TTT TTT	21	6421.2
T	GCT AGG TCC CGT AGT GCG	18	5531.6
3-meT	GCX AGG TCC CGT AGT GCG (X = 3-methylthymine)	18	5545.3

hydrophilic but many will dissolve in relatively high organic solutions and HILIC LC/MS has become of interest to the analysis of large biological molecules including peptides and proteins [40,41]. However, only a very small number of publications describing HILIC LC/MS analysis of oligonucleotides have been published to date probably due to a combination of factors including only a rudimentary understanding of the mechanism of retention. After Alpert's work, Hogrefe et al. [42] used the same column buffered with triethylammonium acetate (TEAA) in HILIC mode for deprotecting methylphosphonate oligonucleotides. Holdšvendová et al. [43] separated three small oligonucleotides on a monolithic column with capillary HILIC chromatography with UV as the detector and buffer was also TEAA. Those three experiments applied HILIC chromatography with UV and MS detection used only for identification. Two used the same polymer based column and one bespoke monolithic column, and all used ion pairing reagent as the buffering system.

In this paper we described a novel method for HILIC LC/MS analysis of synthetic and methylated oligonucleotides using a non-ion-pairing mobile phase containing 'MS friendly' ammonium acetate buffer. It is demonstrated that the method can be used to separate synthetic oligonucleotides 20 mer in length with single nucleotide resolution and shows different selectivity compared to IP-RP LC/MS for oligonucleotide separations. The described method is shown to be capable of purifying and characterizing synthetic and chemically modified oligonucleotides.

2. Experimental

2.1. Chemicals and oligonucleotide samples

Analytical reagent grade ammonium acetate (BDH chemicals, EAST YORKSHIRE, UK), glacial acetic acid (VWR international, West Sussex, UK), and HPLC-grade acetonitrile (ACN) (Fisher Scientific, Leicestershire, UK) were used in all experiments. Water was purified in-house using a Milli-Q system (Millipore, Bedford, MA, USA). The standards of oligonucleotides were purchased as 'standard desalting' from Integrated DNA Technologies (Coralville, IA, USA) without further purification (Table 1).

2.2. Instrumentation

An ESI-TOF mass spectrometer (Micromass® LCT Premier XE, Waters Corporation, Manchester, UK) was coupled with a HP1050 HPLC system (Agilent Technologies UK Ltd., Berkshire, UK) equipped with a degasser, quaternary pump, autosampler and temperature controlled column compartment. The LC/MS system was operated by MassLynx™ software, version 4.1 (Waters Corporation, Milford, MA, USA). LC/MS chromatograms were acquired in negative ion mode using an ESI-MS capillary voltage of 2.5–3.0 kV, a sample cone voltage of 40 V, and an MCP detector voltage of 2400 V. Desolvation gas flow rate was maintained at 800 L/h. Cone gas flow rate was set to 30 L/h. Desolvation temperature and source temperature were set to 450 and 150 °C, respectively. The acquisition range was m/z 700–3500; the 1.1 s cycle consisted of a 1.0 s acquisition time and a 0.1 s delay. Instrument calibration was performed routinely in negative ion mode prior to LC/MS experiment by direct infusion of NaI/RbI (2.0/0.05 μg/μl) in 50/50 2-propanol/water. The mass range for calibration was 200–3500 Da.

2.3. Chromatography

A PEEK ZIC® – HILIC zwitterionic column [100 mm × 2.1 mm (i.d.)] with 3.5 μm particles (average pore diameter 200 Å) (Merck SeQuant, Umeå, Sweden) was used for all LC/MS experiments together with a ZIC® – HILIC 20 mm × 2.1 mm, 5 μm guard column. For gradients, mobile phases, and other conditions, see figure captions.

2.4. Preparation of buffer and oligonucleotide samples

Buffer of 100 mM ammonium acetate was prepared by dissolving 0.7708 g of ammonium acetate in 100 mL of Milli-Q water, and by using glacial acetic acid to bring the pH down to ca. 5.8. All oligonucleotides were used as purchased. Milli-Q water was used to prepare all stock solutions and then diluted by initial mobile phase composition to make work solutions before injected onto column.

Table 2
Evaluation of five HILIC columns.

	ZIC® HILIC	YMC-pack silica	Luna® HILIC	Kinetex® HILIC	ZORBAX HILIC plus
Dimension	2.1 mm × 100 mm, 3.5 μm, 200Å	4.0 mm × 50 mm, 3.0 μm, 200 Å	3.0 mm × 150 mm, 3.0 μm, 200 Å	2.1 mm × 50 mm, 1.7 μm, 100 Å	2.1 mm × 50 mm, 3.5 μm, 95 Å
Gradient	75–50% ACN in 7.5 min	75–30% ACN in 7.5 min	75–50% ACN in 7.5 min	90–50% ACN in 7.5 min	80–55% ACN in 7.5 min
Flow rate (mL/min)	0.5	1.0	0.5	0.2	1.0
Inject amount (picomole)	50	50	50	50	50
Retention time (min)	7.80	3.40	8.05	12.09	5.56
Peak shape	Sharp	Sharp	Sharp	Broad	Broad
Signal to noise (S/N)	67.03	15.64	2.52	1.23	0.85

Fig. 1. The effect of buffer anions and organic modifiers in the mobile phase, on the retention of oligonucleotides. PEEK ZIC® – HILIC, 100 mm × 2.1 mm, 3.5 μm column. Mobile phase A: Milli-Q H_2O; B: acetonitrile – a, b, c, d, methanol – e; C: (a, b, e) 100 mM ammonium acetate, pH 5.8; (c) 100 mM ammonium formate, pH 3.5; (d) 100 mM ammonium bicarbonate, pH 7.6. Gradients (a, b) from 75% to 50% B in 7.5 min, with constant 10% C, flow rate, 0.5 mL/min; (c) from 90% to 50% B in 7.5 min, with constant 10% C, flow rate, 0.6 mL/min but only 0.2 mL/min split to MS; (d) from 80% to 50% B in 7.5 min, with constant 10% C, flow rate, 0.5 mL/min; (e) from 90% to 50% B in 7.5 min, with constant 10% C, flow rate, 0.4 mL/min. Temperature, 30 °C; 50 picomole each injected.

3. Results and discussion

3.1. Method development

Five HILIC columns, each with subtly different stationary phase design, were evaluated with the mobile phases containing constant 10% (by volume) 100 mM ammonium acetate pH 5.8 with ACN gradient (Table 2). Oligonucleotide EVEN was injected onto each column and all five columns produced different retention times for the oligonucleotides, however only the ZIC® HILIC column delivered high signal sensitivity under these conditions. The ZIC® HILIC column was chosen for further investigation as a result of this evaluation. The four remaining columns were not evaluated further in this study but they might be useful for the separation of oligonucleotides under different elution conditions.

Next, the effect of buffer anions in the mobile phase, on the retention of oligonucleotides, was examined using the ZIC® HILIC column. Three volatile buffers commonly used for LC/MS analysis were compared; 100 mM ammonium formate pH 3.5; 100 mM ammonium acetate pH 5.8 and 100 mM ammonium bicarbonate pH 7.6. Fig. 1 clearly shows the different effect of the three buffers. Oligonucleotide T20 was selected for Fig. 1a and c, and EVEN for b, d and e. Ammonium acetate produced the best peak shape and least baseline noise compared to ammonium bicarbonate, however, ammonium formate failed to show any retention for T20 at 50% ACN, the least retentive from A, C, and G rich sequences in HILIC mode [39]. Ammonium acetate was the buffer used in the following experiments in this study, however, ammonium bicarbonate may also be used as an alternative buffer as it showed a good retention for EVEN.

The type of organic modifier was also studied. The solvent strength in HILIC is roughly inverted from what is observed for reversed-phase chromatography. Acetonitrile, one of the weaker solvents in HILIC, provides stronger retention compared to methanol. However, as HILIC chromatography always starts with a very high percentage of organic solvent, it is very important to consider the solubility of buffers in the eluent in HILIC chromatog-

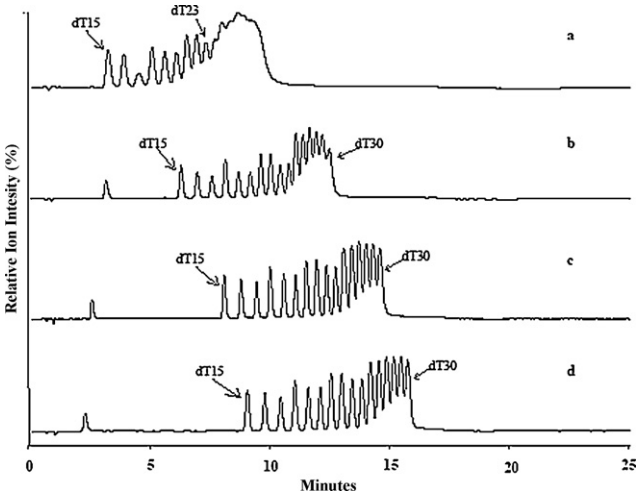

Fig. 2. Separation of oligonucleotides dT15-30. PEEK ZIC® – HILIC, 100 mm × 2.1 mm, 3.5 μm column. Mobile phase A: Milli-Q H_2O; B: acetonitrile; C: 100 mM ammonium acetate, pH 5.8. Gradient from 70% to 60% B in 15 min, with constant C 5% (a), 10% (b), 15% (c) and 20% (d), flow rate, 0.6 mL/min but only 0.2 mL/min split to MS, temperature, 50 °C; 10 picomole each injected.

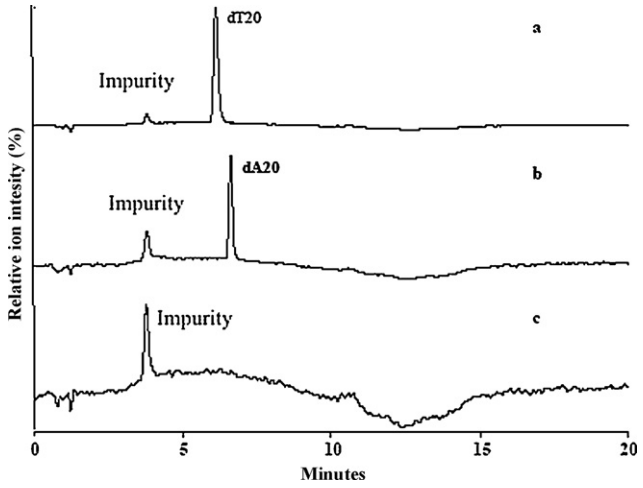

Fig. 3. Retention of homogeneous oligonucleotides. PEEK ZIC® – HILIC, 100 mm × 2.1 mm, 3.5 μm column. Mobile phase A: Milli-Q H_2O; B: acetonitrile; C: 100 mM ammonium acetate, pH 5.8. Gradient from 75% to 50% B in 7.5 min, with constant 10% C, flow rate, 0.6 mL/min but only 0.2 mL/min split to MS, temperature, 50 °C; 50 picomole each injected. (a) dT20, (b) dA20, and (c) dC20.

raphy. Both acetonitrile and methanol enable good solubility of ammonium acetate at the concentrations used in this study [44]. The effect of organic modifiers (methanol and acetonitrile) as weak eluents, on the retention and separation of oligonucleotides, was examined. As seen from Fig. 1e, methanol gave less retention to oligonucleotides with broader peaks and poorer quality of MS spectra than acetonitrile (MS spectra data not shown).

3.2. Separation of homogeneous oligonucleotides

The separation of homooligodeoxythymidines sized from 15 to 30 mer was successfully achieved by a shallow ACN gradient within 15 min (Fig. 2), giving enhanced separation of large oligonucleotides compared to IP-RP-HPLC chromatography buffered with TEA and HFIP [45]. Meanwhile, the HILIC chromatography showed enhanced sensitivity with mass spectrometry, 10 picomole of each oligodeoxythymidine was injected onto the column. The eluent contains a much higher percentage of ACN at the point of oligonucleotide elution from the HILIC column compared to conventional IP-RP-HPLC chromatography. Fig. 2 shows the effect of a higher concentration of ammonium acetate on the separation of larger oligonucleotides; this is the buffer concentration in the mixed mobile phase. The effective concentration of ammonium acetate has to be at least 10 mM to separate homooligodeoxythymidines over 23 mer in length. Higher effective concentration of ammonium acetate showed longer retention suggesting that retention on a ZIC® HILIC column is not only dependent on hydrophilicity of the mobile phase (more hydrophilic mobile phase leading to quicker elution), but also on ionic strength, which may explain why this column shows better selectivity than the others tested in this study under similar conditions. However, very high effective buffer concentrations should be avoided for LC/MS analysis of oligonucleotides as this leads to a reduction in solubility of buffer in the high organic solvent content, and also in sensitivity of the MS signals. The mechanism of the elution trend seen for different buffer concentrations is not understood, but we suggest that the stationary phase of the column may be becoming more hydrophilic through an adsorbtion of buffer ions leading to stronger retention of charged oligonucleotide.

Fig. 3 shows the differences in retention between dT20, dA20 and the absence of dC20 eluting under similar conditions. The first peak in each chromatogram represents impurities in the sample and it can be seen that dC20 did not appear to elute under the HILIC conditions used. Confirmation that dC20 was retained on the column came from its subsequent elution within the following blank injection under higher aqueous conditions (data not shown). This suggests that retention of oligonucleotides in this method is dependent on the sequence, and C rich sequences will be more strongly retained than A rich sequences whilst A rich sequences show slightly stronger retention than T rich sequences. This is consistent with Alpert's observations [39], but the opposite of the trend observed with that of IP-RP-HPLC chromatography [35]. Regrettably, a 20 mer oligodeoxyguanosine was not available. Alpert stated that G rich sequences are more strongly retained than A and T rich sequences using HILIC chromatography [39].

3.3. Separation of heterogeneous oligonucleotides

As stated earlier, the retention behaviors of homooligonucleotides containing A, T, C and G are different from one another, so one should expect heterogeneous oligonucleotides to be much more challenging to separate. However, synthesis of oligonucleotides always produces some impurities such as N – 1 and N – 2 [46] and N + 1 and N + 2 [47], so the analytical method should be capable of separating oligonucleotides from such impurities.

Fig. 4 shows a baseline separation between sequences of EVEN and EVEN G (b), and a good separation between sequences of EVEN and EVEN C (a). The method can be optimized further to gain a maximum resolution between the latter pair. EVEN G and EVEN C have an extra mononucleotide at the 3′ end compared to EVEN, so the method described above is definitely capable of separating oligonucleotides from N + 1 with C or G as the extra mononucleotide. However, one should expect that separation becomes much more challenging when replacing C or G mononucleotide with the less hydrophilic A or T mononucleotide (Fig. 4c and d). As mentioned earlier, HILIC chromatography favors mononucleotides C & G over A & T due to their different hydrophilicity, the former pair is more hydrophilic than the latter so adding a mononucleotide C or G contributes more to the overall hydrophilicity of the sequence than that of A or T, which makes an easier separation of N and N + C/G than that of N and N + A/T. The separation of EVEN from EVEN A and EVEN T can still be possible by increasing the column temperature, as Oefner [48] found that resolution of 16 mer isomeric oligonucleotides differing in a single base with A and T at the 3′-

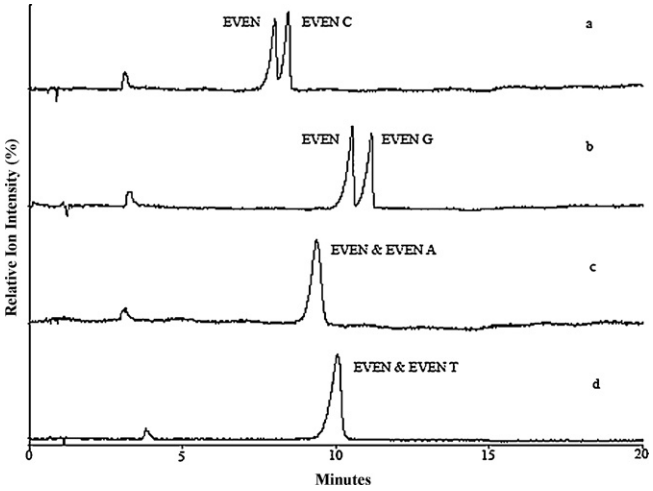

Fig. 4. Separation of heterogeneous oligonucleotides. PEEK ZIC® – HILIC, 100 mm × 2.1 mm, 3.5 μm column. Mobile phase A: Milli-Q H_2O; B: acetonitrile; C: 100 mM ammonium acetate, pH 5.8. Temperature, 50 °C. (a) EVEN and EVEN C, gradient from 65% to 55% B in 10 min, with constant 10% C, flow rate, 0.5 mL/min; (b) EVEN and EVEN G, gradient from 65% to 55% B in 10 min, with constant 15% C, flow rate, 0.4 mL/min. (c) EVEN and EVEN A, gradient from 65% to 55% B in 10 min, with constant 10% C, flow rate, 0.5 mL/min; (d) EVEN and EVEN T, gradient from 65% to 55% B in 10 min, with constant 10% C, flow rate, 0.4 mL/min. 100 picomole of EVEN, EVEN C and EVEN T each injected, 200 picomole of EVEN G and EVEN A each injected.

end improves significantly with an increase in column temperature from 50 to 80 °C. Due to the temperature restriction of the silica based ZIC® HILIC column (maximum 70 °C), a column temperature of 50 °C was used for all oligonucleotide separations in this study. Oefner demonstrated that higher temperature can produce increased resolution for the separation of oligonucleotides using IP-RP-HPLC [48]. Higher temperatures may produce improved results using this silica-based column but this was not evaluated in the current experiments in the interest of long-term column performance.

After changing the arrangement of bases for EVEN, i.e. NEW EVEN, the method described above can fully separate NEW EVEN from N + A/T/C/G (Fig. 5). One may notice that the separation

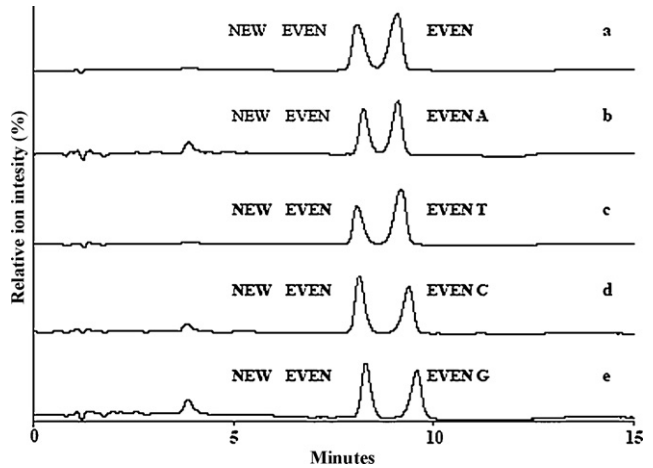

Fig. 5. Separation of heterogeneous oligonucleotides. PEEK ZIC® – HILIC, 100 mm × 2.1 mm, 3.5 μm column. Mobile phase A: Milli-Q H_2O; B: acetonitrile; C: 100 mM ammonium acetate, pH 5.8. Gradient from 65% to 55% B in 7.5 min, with constant 10% C, flow rate, 0.4 mL/min, temperature, 50 °C. 100 picomole each injected except for EVEN A and EVEN G, 200 picomole each injected. (a) NEW EVEN & EVEN; (b) NEW EVEN & EVEN A; (c) NEW EVEN & EVEN T; (d) NEW EVEN & EVEN C; (e) NEW EVEN & EVEN G.

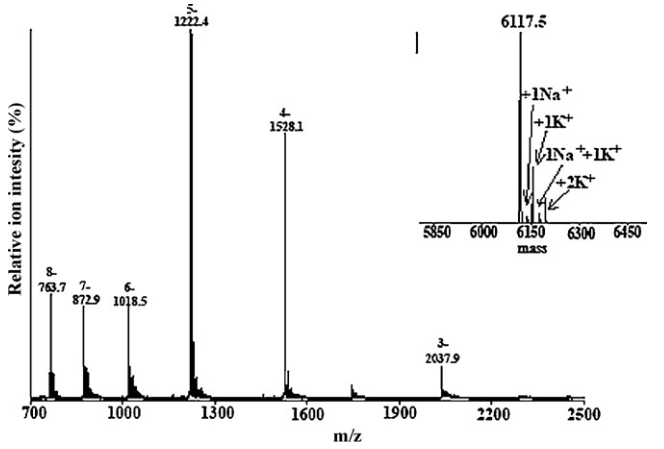

Fig. 6. Mass spectrum of NEW EVEN. Inset to figure is deconvoluted spectrum of NEW EVEN.

of oligonucleotides is dependent on the base sequence. The same method struggled to separate EVEN A and EVEN T from EVEN, but the two sequences can be well separated from NEW EVEN. One can understand that sequences containing different amounts of mononucleotides A, T, C & G will have differing retention on the HILIC column because their individual differences in hydrophilicity will contribute to overall differences in hydrophilicity of the full oligonucleotide sequence. However, sequences EVEN and NEW EVEN contain exactly the same amount of mononucleotides A, T, C & G but differ in their arrangement throughout the molecule demonstrating that the retention behavior of heterogeneous oligonucleotides is not only dependent on the proportion of mononucleotides but also on the arrangement of those four mononucleotides using this method.

Fig. 6 shows the mass spectrum of NEW EVEN and its deconvoluted spectrum. As seen from Fig. 6, the method described did not produce a very complex mass spectrum of oligonucleotide, which certainly helped data interpretation and improved mass measurement accuracy of oligonucleotide.

3.4. LC/MS analysis of methylated oligonucleotides

We have shown that the method presented is capable of separating synthetic oligonucleotides from the impurities that originated from the processes of synthesis. However, an LC/MS method may also be required to separate modified oligonucleotides where modifications can be positioned at the 3′- or 5′-end of the oligonucleotide, or internally within the sugar-phosphate backbone or on nucleobases. Gilar [35] used IP-RP-HPLC to separate a 21 mer TaqMan oligonucleotide probe labeled at the 5′ and 3′ ends with fluorescein and TAMRA, respectively. Bothner et al. [49] applied RP-LC/MS to the separation of two oligonucleotides containing a mixture of phosphodiester and methylphosphonate bonds. Fig. 7 illustrates the separation of a 18 mer oligonucleotide (T) and its methylated product containing a 3-methylthymine (3-meT). Methylation of a thymine base reduces the overall hydrophilicity of the oligonucleotide to a small extent, enabling the separation of an oligonucleotide from its methylated product. As seen from Fig. 7, 3-meT was less retained compared to T.

3.5. Regeneration of ZIC® HILIC column

It was noticed that after a significant number of oligonucleotide injections on the column (over 200), the performance of the ZIC® HILIC column started to change. The sensitivity of the MS signal started to reduce and retention times slightly decreased. No change

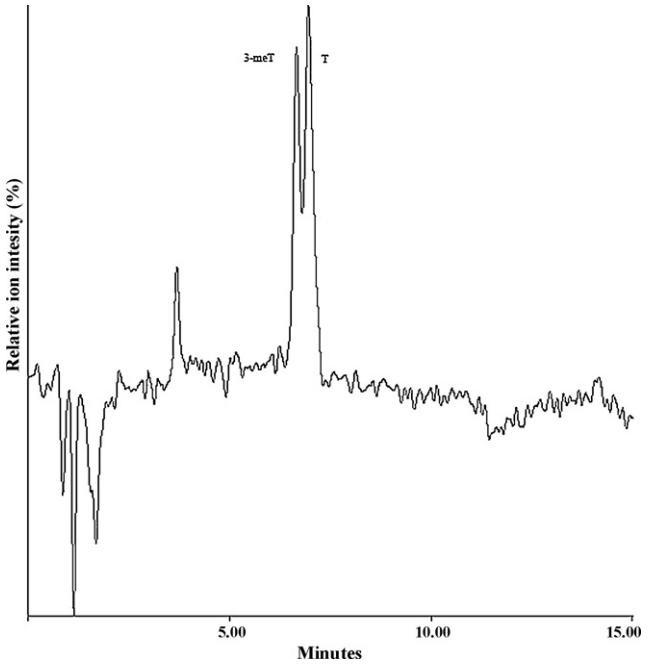

Fig. 7. LCMS of a mixed 18 mer and its methylated oligonucleotide. PEEK ZIC® – HILIC, 100 mm × 2.1 mm, 3.5 μm column. Mobile phase A: Milli-Q H_2O; B: acetonitrile; C: 100 mM ammonium acetate, pH 5.8. Gradient from 65% to 55% B in 7.5 min, with constant 10% C, flow rate, 0.4 mL/min, temperature, 50 °C. 100 picomole each injected.

to relative selectivity was seen and the system backpressure stayed the same. We believe that continuous elution over 50% organic solvent does not completely remove all oligonucleotides and impurities from the HILIC column, and a small amount (mainly anions) remain bound to the quaternary ammonium group of the stationary phase leading to loss of performance over time. According to the instruction of the ZIC® HILIC column manufacturer, separation on the ZIC® HILIC column is achieved by a hydrophilic partitioning mechanism with weak electrostatic interactions. Changing the overall charges of the stationary phase would therefore affect the performance of the ZIC® HILIC column. Higher buffer concentration in the mobile phase can be used to reduce the effect of electrostatic interactions but may reduce MS sensitivity. A higher aqueous composition in the mobile phase is efficient at washing off those strongly bounded analytes and impurities, but to do this for each injection would be time-consuming and it was decided to regenerate the ZIC® HILIC column periodically to regain the column performance.

We regenerated the ZIC® HILIC column successfully by the following procedure:

1. Washing the column with enough water;
2. Washing the column with 30 column volumes of 50:50 (v/v) H_2O:ACN;
3. Washing the column with 30 column volumes of water;
4. Flushing the column with 30 column volumes of 200 mM ammonium acetate pH 5.8;
5. Washing the column with 30 column volumes of water;
6. Conditioning the column with initial LC conditions.

An alternative approach would be to incorporate these steps into the method for each run however our experience has been that this is a less efficient approach considering the relatively large number of injections afforded before regeneration is necessary. There may be other ways to regenerate the column but these were not investigated.

4. Conclusions

A novel HILIC based HPLC ESI-TOFMS method was developed for the characterization and identification of synthetic and chemically modified oligonucleotides. Using a PEEK ZIC® – HILIC zwitterionic column [100 mm × 2.1mm (i.d.)] with 3.5 μm particles (average pore diameter 200 Å) with gradients of acetonitrile buffered with an effective concentration of 10 mM ammonium acetate pH 5.8, synthetic oligonucleotides 20 mer in length can be separated within 7.5 min with single nucleotide resolution. The chromatography enables efficient desalting and alkali cation adduction was very low, allowing for accurate mass determination. The impact of the oligonucleotide sequence (nucleobase composition) on the separation of the target product from its failure sequences was studied, and it was found that HILIC chromatography produces stronger retention of C and G rich sequences than A and T rich sequences. The majority of oligonucleotides can be eluted from the HILIC column by 50% acetonitrile (or higher) with ammonium acetate, an 'MS friendly' buffer, which leads to increased MS sensitivity. MS detection limits were in the lower picomole range with full-scan mode making the method highly capable of quickly and sensitively identifying and resolving, synthetic and chemically modified, oligonucleotides.

References

[1] J. Sambrook, D. Russell, Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
[2] H.Y. Wang, R.L. Malek, A.E. Kwitek, A.S. Greene, T.V. Luu, B. Behbahani, B. Frank, J. Quackenbush, N.H. Lee, Genome Biol. 4 (2003) R5.
[3] M. Scully, J. Brown, R. Patel, V. McDonald, C.J. Brown, S. Machin, J. Thromb. Haemost. 8 (2010) 257.
[4] H.F. Yin, H. Moulton, C. Betts, M. Wood, Methods Mol. Biol. 683 (2011) 321.
[5] M. Aarts, H. Te Riele, Gene Ther. 18 (2011) 213.
[6] E.W. Harkins, Current Protocols in Nucleic Acid Chemistry, John Wiley and Sons Inc., 2001.
[7] M.P. Henry, in: W.S. Hancock (Ed.), High-performance Liquid Chromatography in Biotechnology, Wiley, New York, 1990.
[8] P. Courver, C. Malvy, Pharmaceutical Aspects of Oligonucleotides, Taylor and Francis, London, 2000.
[9] R. Hecker, D. Riesner, J. Chromatogr. B: Biomed. Sci. Appl. 418 (1987) 97.
[10] J.A. Thompson, R.D. Wells, Nature 334 (1988) 87.
[11] K.I. Kasai, J. Chromatogr. B: Biomed. Sci. Appl. 618 (1993) 203.
[12] C.G. Huber, E. Stimpfl, P.J. Oefner, G.K. Bonn, LC–GC Int. 14 (1996) 114.
[13] Y.Z. Xu, P.F. Swann, Anal. Biochem. 204 (1992) 185.
[14] E.M. Southern, Anal. Biochem. 100 (1979) 319.
[15] P.G. Righetti, C. Gelfi, M. Perego, A.V. Stoyanov, A. Bossi, Electrophoresis 18 (1997) 2145.
[16] G. Schomburg, in: N.A. Guzman (Ed.), Capillary Electrophoresis Technology, Chromatographic Science Series, vol. 64, Marcel Decker Inc., New York, 1993.
[17] A. Guttman, K. Ulfelder, Adv. Chromatogr. 38 (1998) 301.
[18] M. Stano, H.D. Flosadottir, O. Ingolfsson, Rapid Commun. Mass Spectrom. 20 (2006) 3498.
[19] H. Tsubery, M. Mrksich, Langmuir 24 (2008) 5433.
[20] Z. Cui, A. Jacob, A. Farrel, A. Burdzy, L.C. Sowers, Anal. Biochem. 379 (2008) 196.
[21] J.R. Thayer, N. Puri, C. Burnett, M. Hail, S. Rao, Anal. Biochem. 399 (2010) 110.
[22] J. Anichina, Z. Dobrusin, D. Bohme, J. Phys. Chem. B 114 (2010) 15106.
[23] Q. Liao, N.H.L. Chiu, C. Shen, Y. Chen, P. Vouros, Anal. Chem. 79 (2007) 1907.
[24] S.P. Hong, S.K. Soo, E.H. Lee, E.O. Kim, S.I. Ji, H.J. Chung, S.N. Park, W. Yoo, W.R. Folk, S.O. Kim, Nat. Protoc. 3 (2008) 1476.
[25] B. Boontha, J. Nakkuntod, N. Hirankarn, P. Chaumpluk, T. Vilaivan, Anal. Chem. 80 (2008) 8178.
[26] J.T. Stults, J.C. Marsters, Rapid Commun. Mass Spectrom. 5 (1991) 359.
[27] X. Cheng, D. Gale, H.R. Udseth, R.D. Smith, Anal. Chem. 67 (1995) 586.
[28] C.G. Huber, A. Krajete, J. Mass Spectrom. 35 (2000) 870.
[29] C. Liu, Q. Wu, A.C. Harms, R.D. Smith, Anal. Chem. 68 (1996) 3295.
[30] C.G. Huber, M.R. Buchmeiser, Anal. Chem. 70 (1998) 5288.
[31] C.L. Andrews, P. Vouros, A. Harsch, J. Chromatogr. A 856 (1999) 515.
[32] H.C. Box, H.B. Patrzyc, J.B. Dawidzik, H. Iijima, H.G. Freund, A.J. Higbee, E.E. Budzinski, Radiat. Res. 158 (2002) 538.
[33] B. Lei, S. Li, L. Xi, J. Li, H. Liu, X. Yao, J. Chromatogr. A 1216 (2009) 4434.
[34] S. Li, D.D. Lu, Y.L. Zhang, S.Q. Wang, Chromatographia 72 (2010) 215.
[35] M. Gilar, Anal. Biochem. 298 (2001) 196.
[36] P. Deng, X. Chen, G. Zhang, D. Zhong, J. Pharm. Biomed. Anal. 52 (2010) 571.
[37] M. Gilar, K.J. Fountain, Y. Budman, U.D. Neue, K.R. Yardley, P.D. Rainville, R.J. Russell, J.C. Gebler II, J. Chromatogr. A 958 (2002) 167.
[38] A. Apffel, J.A. Chakel, S. Fischer, K. Lichtenwalter, W.S. Hancock, Anal. Chem. 69 (1997) 1320.

[39] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[40] B.Y. Zhu, C.T. Mant, R.S. Hodges, J. Chromatogr. 548 (1991) 13.
[41] A.R. Oyler, B. Armstrong, J.Y. Cha, M.X. Zhou, Q. Yang, R.I. Robinson, et al., J. Chromatogr. A 724 (1996) 378.
[42] R.I. Hogrefe, M.M. Vaghefi, M.A. Reynolds, K.M. Young, L.J. Arnold, Nucleic Acids Res. 21 (1993) 2031.
[43] P. Holdšvendová, J. Suchánková, M. Bunček, V. Bačkovská, P. Coufal, J. Biochem. Biophys. Methods 70 (2007) 23.
[44] A.P. Schellinger, P.W. Carr, LCGC N. Am. 22 (2004) 544.
[45] K.J. Fountain, M. Gilar, J.C. Gebler, Rapid Commun. Mass Spectrom. 17 (2003) 646.
[46] D. Chen, Z. Yan, D.L. Cole, G.S. Srivatsa, Nucleic Acids Res. 27 (1999) 389.
[47] A.H. Krotz, P.G. Klopchin, K.L. Walker, G.S. Srivatsa, D.L. Cole, V.T. Ravikumar, Tetrahedron Lett. 38 (1997) 3875.
[48] P.J. Oefner, J. Chromatogr. B 739 (2000) 345.
[49] B. Bothner, K. Chatman, M. Sarkisian, G. Siuzdak, Bioorg. Med. Chem. Lett. 5 (1995) 2863.