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Electrospray ionization mass spectrometric analysis of nucleic acids using high-throughput on-line desalting

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A rapid on-line desalting method utilizing ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) was employed in tandem with negative electrospray ionization mass spectrometry (ESI-MS) for the routine analysis of nucleic acids. Desalting was performed on a short 10 × 2.1 mm guard column packed with 3.5 μm C_{18} sorbent. The HPLC system was connected in-line to an orthogonal ESI-TOF mass spectrometer via a six-port, two-position switching valve, allowing desalting followed by mass analysis of nucleic acids. Duty cycle times for the method were as low as 1.5 min per sample. This allowed for the analysis of approximately 950 samples per 24-h time period, which is suitable for medium- to high-throughput applications. Average mass accuracy was determined to be 80 ppm for oligonucleotides up to 110 mer in length with external calibration. The method was utilized for synthetic oligonucleotide quality control and analysis of DNA genotyping fragments. Copyright © 2004 John Wiley & Sons, Ltd.

Mass spectrometry (MS) is a useful analytical technique for the analysis of nucleic acids, due to its inherent ability to identify molecules based on their respective molecular masses, which directly correlates to their molecular makeup. More specifically, MS has been employed in the areas of deoxyribonucleic acid (DNA) genotyping,[1,2] synthetic and therapeutic oligonucleotide quality control (QC),[3,4] and polymerase chain reaction (PCR) product analysis.[5,6] Due to the broad application of nucleic acids in numerous fields, it is imperative to develop fast and simple methods for their analysis.

Traditionally, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) has been used for high-throughput analysis of DNA.[7,8] Cycle times have been shown to be as low as a few seconds per sample, allowing over 20 000 samples to be analyzed per day.[9] However, this impressive throughput does not include the time required for sample preparation. Further, oligonucleotides greater than 50 mer in length are challenging to analyze by MALDI, due to decreasing signal sensitivity and mass accuracy.

The affinity of the DNA backbone for alkali cation adduction (Na^+, K^+) further complicates analysis by MS, due to the formation of multiple signals for each charge state. Sample desalting is crucial for successful nucleic acid identification and characterization. Several off-line sample preparation methods have been found to be effective;[10–14] however, these methods are labor-intensive and add significant time to the overall duty cycle, which undermines the advantage of the throughput of MALDI-TOFMS.

Electrospray ionization mass spectrometry (ESI-MS) has also been utilized for the investigation of nucleic acids. The formation of multiply charged ions allows for the analysis of large DNA fragments. ESI-MS can be easily interfaced on-line with reversed-phase high-performance liquid chromatography (RP-HPLC).

DNA desalting methods can be performed on- or off-line. On-line methods are usually sequential in nature, with only one sample being run at a time. Currently available HPLC systems can be easily adapted for on-line desalting due to the prospects of automation, minimal sample manipulation, and inexpensive operation. RP-HPLC is the on-line method of choice because contaminating salts flow through the column while the DNA of interest is retained, and can subsequently be eluted directly to a mass spectrometer. One of the challenges for interfacing HPLC on-line with ESI-MS for nucleic acid analysis is finding MS-compatible mobile phases that do not cause ion suppression and/or substantial background noise.[4,15,16] Additional published reports have coupled ion-pair reversed-phase HPLC (IP-RP-HPLC) on-line with ESI-MS in order to achieve separation and characterization of DNA-based drugs.[17–20] Although the speed of LC/MS analysis is acceptable for characterization of therapeutic oligonucleotides, many applications (i.e. synthetic oligonucleotide QC and genotyping) require more rapid sample throughput.

Faster analysis times (6–10 min/sample) have been reported using IP-RP-HPLC coupled to ESI-MS.[16,21–24] Other reports[19,25,26] utilizing RP-HPLC with on-line ESI-MS detection for analysis of phosphorothioate (PS) oligonucleotides and PCR products achieved comparable analysis times with similar results. In order to achieve even faster analysis times, HPLC has been used only for desalting prior to elution to ESI-MS. Huber and Buchmeiser[27] utilized on-line cation-exchange microcolumns to trap salt contaminants while allowing nucleic acids to flow through for ESI-MS analysis.

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Liu *et al*.[28] and Xu *et al*.[29] employed on-line microdialysis for oligonucleotide desalting prior to ESI-MS analysis. The method was efficient for desalting ssDNA up to 34 mer, with duty cycle times of ~5 min/sample. While the methods described above proved to be very sensitive and robust for desalting and identification of both single- and double-stranded DNA, duty cycle times remain insufficient for high-throughput applications.

In this paper, the development of a method for the high-throughput on-line desalting of nucleic acids prior to ESI-MS analysis is described. The method is fast and simple, and is generic for all nucleic acid samples. The RP-HPLC column is utilized only as a desalting tool; all sample components were eluted with a step gradient as a single peak and analyzed by the mass spectrometer. Duty cycle times of 1.5 min/sample were routinely achieved, which is suitable for medium- to high-throughput applications. The method was applied for the quality control (QC) of synthetic oligonucleotides and DNA genotyping.

EXPERIMENTAL

Chemicals and oligonucleotide samples

Glacial acetic acid (99.99%) and HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from J. T. Baker (Phillipsburg, NJ, USA). Water (18 MΩ cm) was purified in-house using a Milli-Q® system (Millipore, Bedford, MA, USA). Triethylamine (TEA; 99%), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), *N,N*-dimethylbutylamine (99%), and 3-hydroxypicolinic acid (3-HPA) were all purchased from Sigma (St. Louis, MO, USA). Synthetic oligonucleotides ranging in size from 24–110 mer were supplied in high purity, salt-free form by MWG Biotech (High Point, NC, USA). All oligonucleotide sequences not used for PCR were comprised of a 5′-GAC TTA-3′ repetitive motif unless otherwise specified.

Polymerase chain reactions

All polymerase chain reaction (PCR) experiments were performed using a PTC-100™ thermal cycler (MJ Research™ Inc., Waltham, MA, USA). Reactions for single nucleotide polymorphism (SNP) genotyping were performed according to the published protocol.[30] Briefly, each 20-μL reaction consisted of 5.1 mM $MgCl_2$, 1.3× GeneAmp PCR Gold Buffer (10× stock), 1.04 U AmpliTaq Gold DNA polymerase (all from Applied Biosystems, Framingham, MA, USA), 0.48 mM dNTPs (Epicentre Technologies, Madison, WI, USA), 0.29 μM each primer (MWG Biotech), and 40 ng genomic DNA template (Coriell Institute, Camden, NJ, USA). Nuclease-free water (Ambion Inc., Austin, TX, USA) was used to bring each reaction to 20 μL total volume. Initial denaturation was performed at 94°C for 10 min, followed by 12 cycles of denaturation at 94°C for 15 s, annealing at 65°C for 15 s (−0.5°C each cycle), and extension at 72°C for 50 s. This was followed by an additional 30 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s, and extension at 72°C for 50 s. Finally, linear amplification was performed at 60°C for 40 min after the addition of 5 μL of the following mixture: 1.6× ThermoPol buffer (10× stock), 1.6× N.BstNB I buffer (10× stock), 20.8 U of N.BstNB I nicking enzyme, 2.1 U Vent(exo-) (all from New England Biolabs, Beverly, MA, USA), 0.7 mM dNTPs (Epicentre Technologies), and nuclease-free water. The end result was the generation of short single-stranded oligonucleotides (8–16 mer) containing the SNP of interest.

Primers were obtained in high-purity, salt-free form from MWG Biotech. Primers are labeled according to either the Ionian Technologies[31] (Upland, CA, USA) or SNP Consortium[32] (TSC) database. Sequences were as follows (5′ to 3′): PG58320 forward primer TGT CCC TAA ATC TGG TGA GTC AGC CTC; PG58320 reverse primer CTG TTA AAG GAT GAT CAC GAG TCC ACA GA; HGBASE forward CCG AGG TAT GGC AGA GAG TCA AGA CC; HGBASE reverse GCC TCC TAG GCA GTG AGT CAG AGG A; BDNF-V66M forward TGA CAT CAT TGG CTG AGT CTT TCG A; BDNF-V66M reverse TTC TGG TCC TCA TCC AAG AGT CCT TCT A; NAT1 forward AGT CTA TGA ATA CAT ACC TGA GTC CAT CTC; NAT1 reverse ACA AAA TGA TTT ACG AGT CAA CAC A; IL1B forward ACA TGT GCT CCA CAT TTC AGA GTC TAT CTT; IL1B reverse GCA CAT AAG CCT CGG AGT CCC ATG T; IL8 forward TGA TAA AGT TAT CTA GGA GTC AAA AAG CAT; IL8 reverse CTG AAG CTC CAC AAG AGT CTG AAT TAT C. Human genomic DNA samples purchased from the Coriell Institute are labeled NA15029, NA15221, and NA15506.

On-line desalting system configuration

An Alliance® 2795 HPLC system (Waters Corporation, Milford, MA, USA) served as both the autosampler and elution pump. Elution flow rate was 0.3 mL/min. Experiments were performed at ambient temperature. Either a 20 × 2.1 mm or 10 × 2.1 mm, 3.5 μm Xterra® MS C_{18} guard column (Waters) was used for sample desalting. A 515 HPLC pump (Waters) was plumbed through the Alliance 2795 HPLC system to serve as the load/wash pump. Load/wash flow rate was 0.9 mL/min. Salts eluting from the column were diverted to waste via a switching valve during the load/wash phase of the LC/MS method. Load/wash buffer consisted of 5% ACN and 95% 5 mM dimethylbutylammonium acetate (DMBAA), pH 7. Elution mobile phase consisted of 25% ACN and 75% 5 mM DMBAA, pH 7. The configuration of the system is discussed in greater detail in the Results and Discussion section.

Mass spectrometry

An orthogonal ESI-TOF mass spectrometer (Micromass® LCT™, Waters) was connected in-line to the HPLC system and isocratic pump via a Rheodyne® six-port, two-position, stainless steel switching valve (Waters). LC/MS chromatograms were acquired in negative ion mode using an ESI-MS capillary voltage of 2.5 kV, a sample cone voltage of 25 V, an extraction cone voltage of 1 V, and an MCP detector voltage of 2700 V. Desolvation gas flow rate was maintained at 410 L/h. Cone gas flow rate was set to 30 L/h. Desolvation temperature and source temperature were set to 275 and 100°C, respectively. Acquisition range was *m/z* 500–2000. The 0.95 s scan cycle consisted of a 0.9 s acquisition time and a 0.05 s delay. Instrument calibration was performed routinely in negative ion mode prior to LC/MS experiments by direct infusion of poly-DL-alanine (Sigma, St. Louis, MO, USA) in

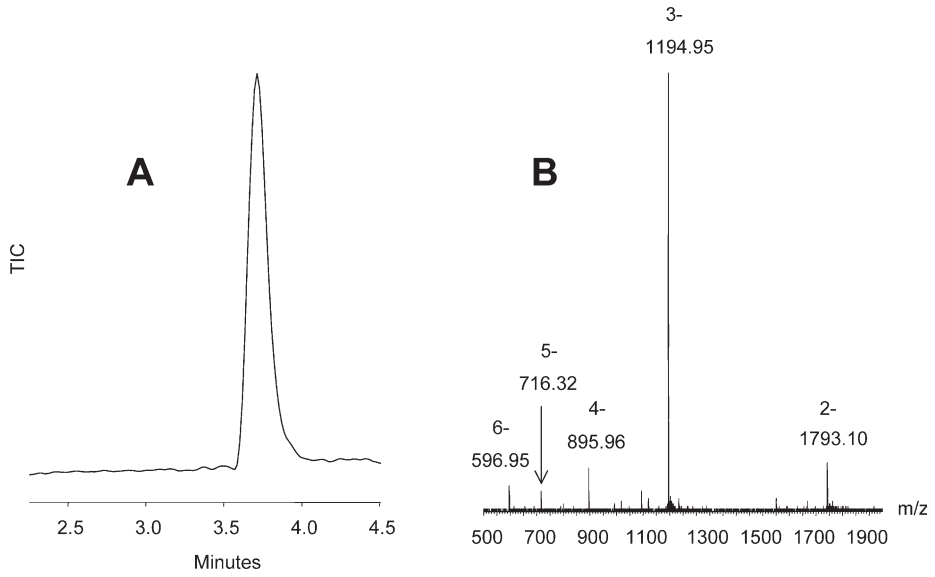

Figure 1. Total ion chromatogram (A) and raw spectrum (B) from the LC/MS analysis of a 12mer oligodeoxythymidine ($d(T)_{12}$). A ballistic gradient was used for desalting prior to ESI-TOFMS analysis. XTerra® MS C_{18}, 20 × 2.1 mm, 3.5 μm column. Mobile phase A: 5 mM DMBAA, pH 7.0, B: 50% ACN in 5 mM DMBAA, pH 7.0. Gradient from 10–30% B in 2.5 min, followed by 30–90% B in 0.5 min. First 2.25 min of analysis diverted to waste. Re-equilibration performed for 1.5 min at 0.3 mL/min, 30°C.

50% methanol/50% water at 10 μL/min. The mass range for calibration was as specified above. The LC/MS system was operated by the manufacturer's software. Raw summed spectra were deconvoluted using the MaxEnt1™ software.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was performed using a Micromass® M@LDI™ *LR* (Waters Corporation, Milford, MA, USA) in the positive linear mode. Pulse voltage was set to 1200 V, source 15 000 V, MCP 1850 V, matrix suppression delay was set to 500 amu. The instrument was operated by the manufacturer's software. Matrix for all MALDI analyses was comprised of 25 mg of 3-HPA dissolved in 1 mL of 25% ACN/75% water. The 3-HPA matrix was rinsed with 100% acetone and desalted with Dowex ion-exchange (IEX) beads (NH_4^+ form) prior to dissolution in 25% ACN/75% water. The analyte was mixed 1:1 with matrix, and 1 μL was spotted on a stainless steel MALDI target for subsequent analysis.

RESULTS AND DISCUSSION

On-line HPLC desalting system optimization

Previously published methods for analysis of synthetic oligonucleotides and SNP genotyping fragments utilized IP-RP-HPLC interfaced on-line with ESI-TOFMS for the separation and characterization of the target compound(s) from impurities in the mixture.[17,18] The use of capillary columns for these methods allowed for sensitive analysis and low sample consumption. While these methods prove very useful in a low-throughput environment, the duty cycle times of 10–40 min/sample are not desirable for routine desalting experiments.

The LC/MS method was modified to improve the speed of analysis by means of a steep linear gradient (5–15% ACN in 2.5 min). A short 20 × 2.1 mm column was used to retain the injected sample; unretained salts and/or buffer components were diverted to waste for the first 2.25 min of analysis. The gradient eluted the desalted oligonucleotides directly to the mass spectrometer for mass analysis. Analysis of a 12 mer oligodeoxythymidine ($(dT)_{12}$) using this method is shown in Fig. 1. The total ion chromatogram (TIC, Fig. 1(A)) shows elution of the target component at about 3.75 min. Summation of the portion under this peak gives the raw spectrum in Fig. 1(B), where five different charge states for the target oligonucleotide were observed. Little alkali ion adduction was observed.

The total analysis time, including column regeneration, equilibration and sample injection, was 6.5 min, which is not considered high-throughput. Closer examination of the duty cycle (Table 1) reveals that the gradient generation and column equilibration are the main contributing factors to the overall duty cycle time. This is further complicated by the

Table 1. Comparison of duty cycle times for rapid LC/MS analysis of nucleic acids

Method	1	2	3
Column size (L × i.d.)	20 × 2.1 mm	20 × 2.1 mm	10 × 2.1 mm
Column V_0 (μL)	44.1	44.1	22.1
Load/wash flow (mL/min)	0.6	0.9	0.9
Load/wash time (min)	*1.00*	*0.50*	*0.25*
Elution flow (mL/min)	0.3	0.3	0.3
Elution time (min)	*1.00*	*1.00*	*0.88*
Equilibration time (min)[a]	*1.00*	*0.50*	*0.37*
Injection time (min)	0.72	0.72	0.72
Injection mode	Parallel	Parallel	Parallel
Total duty cycle time	*3.0*	*2.0*	*1.5*

[a] Equilibration time was incorporated into sample injection time. This parameter is inherent to the HPLC system, and cannot be controlled by the operator.

gradient delay, which is defined as the time lag between the gradient start and the moment when it actually reaches the column. For example, in the method described above, the gradient starts at time zero (immediately after injection) and ends at 2.5 min. However, the peak of interest was observed eluting at 3.75 min, the point when the HPLC pump was actually delivering the equilibration mobile phase (5% ACN and 95% 5 mM DMBAA, pH 7). The gradient delay is related to the volume of the gradient mixer and tubing, ranging from tens to thousands of microliters (depending on the HPLC system), and cannot be completely eliminated.

In order to achieve faster duty cycle times, a step gradient was evaluated. This was generated by two separate isocratic pumps plumbed through a six-port, two-position switching valve (Fig. 2). In this setup, the injected sample is first loaded onto the column with the load/wash solvent delivered by the load/wash pump (Fig. 2(A)). Salts and unwanted components are diverted from the column outlet to waste by washing for 15 s with the load/wash solvent. When the valve is switched to the elution position, retained analytes (oligonucleotides) are instantaneously eluted from the column to the mass spectrometer by the elution mobile phase (25% ACN and 75% 5 mM DMBAA, pH 7), which is delivered by the second isocratic pump (elution pump, Fig. 2(B)). After elution, the valve is switched back to the load position and equilibrated with the load/wash mobile phase from pump one (load/wash pump). The advantage of this setup is that there is essentially no gradient delay. The load, wash, elution, and equilibration times are now related to the void volume of the column, injector, sample loop, and connective tubing.

Using the setup shown in Fig. 2, the rapid desalting method was designed with either a 20×2.1 mm or 10×2.1 mm column. The wash and elution times necessary for complete desalting and elution were evaluated experimentally. It was found that at least 10 column void volumes (V_0) of mobile phase were necessary to completely desalt or elute the retained sample. For this reason, it is beneficial to use a shorter column with a smaller V_0 in order to achieve faster duty cycle times (Table 1). Thus, using a 10-mm column instead of a 20-mm column decreased the load/wash time by half (compare methods 1 and 2 with method 3 in Table 1). An increase in flow rate also causes the load/wash time to decrease, as the 10 column volumes necessary for complete desalting/elution/equilibration are achieved in a shorter time. No sample breakthrough was detected with either column length (10 or 20 mm) during the load and wash steps.

The choice of buffers is important for the success of on-line desalting prior to ESI-MS. The 5 mM aqueous DMBAA ion-pairing buffer, pH 7, was selected in order to enhance nucleic acid retention during the load/wash step. The addition of 5% ACN to the load/wash buffer was helpful for the cleanup of genotyping samples, reducing the amount of contaminants and surfactants from PCR buffers. The DMBAA aqueous buffer with 25% ACN was used for elution. Using higher concentrations of ACN in the elution buffer resulted in broader peaks and reduced oligonucleotide recovery. This may be associated with the limited solubility of nucleic acids in organic solvents. No MS signal suppression was observed using DMBAA buffers in the elution mobile phase.

Three different configurations of the rapid LC/MS method were evaluated by varying the load/wash, elution, and equilibration timing and flow rates (Table 1). The duty cycle time achieved for a 10×2.1 mm column ($V_0 \sim 22.1$ μL) was as short as 1.5 min. Further progress is limited by the speed of the autosampler. Adjusting injection parameters (needle wash volume, sample draw speed, etc.) and using a parallel injection type reduced the autosampler injection time. Parallel injection mode allows a sample to be introduced into the sample loop while the previous analysis is running. Additionally, the equilibration time is incorporated into the

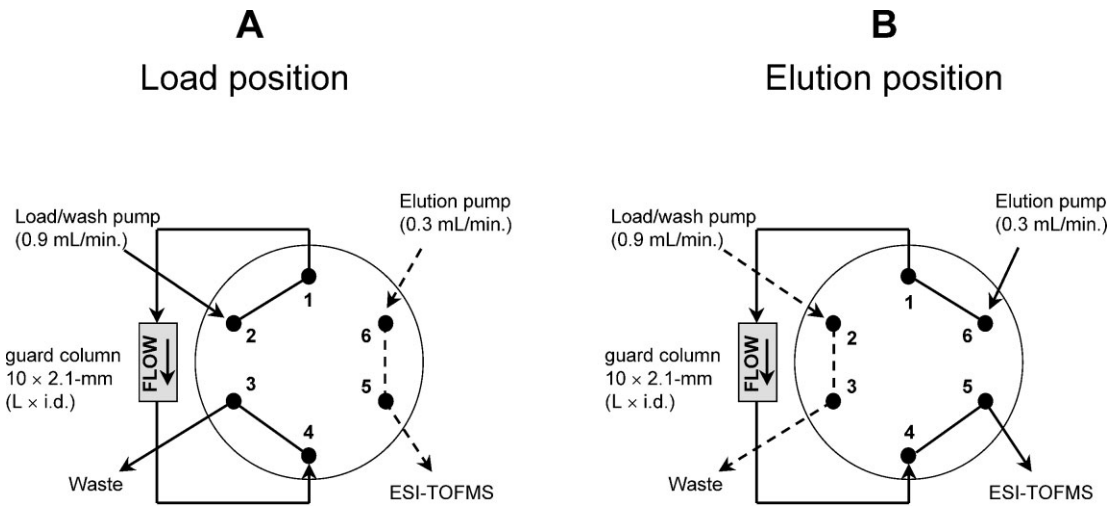

Figure 2. Configuration of two-position, six-port switching valve for rapid on-line desalting of nucleic acids. The load position (A) was used for loading and washing the sample, and for re-equilibration of the column prior to the next analysis. The elution position (B) was simply utilized to elute all retained components to the ESI-TOF mass spectrometer. The solid lines in both (A) and (B) indicate the flow path through the column. The duty cycle consisted of 15 s of loading/washing (load position), 53 s elution (elution position), and 22 s re-equilibration (load position). For system, mobile phase, and column information, see Experimental section.

end of each run while the sample is being loaded onto the column for injection.

Faster on-line desalting prior to MS analysis can be achieved with a faster autosampler and two HPLC guard columns that alternate between the load and elution positions (ten-port, two-position switching valve required). The results discussed in the following sections were acquired using method 3 shown in Table 1.

Application to synthetic oligonucleotide QC

Manufacturers of synthetic oligonucleotides often utilize MS as a qualitative QC tool. Analysis of oligonucleotides by either ESI- or MALDI-MS gives molecular weight confirmation of the target product, and can also indicate a major failure in the synthesis. However, oligonucleotides greater than 50 mer in length become more difficult to analyze by MS, due to decreasing purity and increasing affinity for salt contaminants.

In our experience, even oligonucleotides synthesized in 'high-purity and salt-free' form still contain traces of alkali cations. This is demonstrated in Fig. 3(A), which shows the flow injection analysis (FIA) of a 55 mer synthetic oligonucleotide (samples were reconstituted in 50% deionized water/50% ACN and injected into the ESI-TOF instrument). Multiple sodium adducts were observed, which complicates the mass spectrum (Fig. 3(A)). When the same oligonucleotide (reconstituted in deionized water only) was injected onto the optimized LC/MS system, dramatic improvement of the MS signal quality for the target 55 mer was observed (Fig. 3(B)). This indicates that the LC/MS method is efficient for on-line oligonucleotide desalting.

The method was further employed for the cleanup of synthetic oligonucleotides in the 25–110 mer range prior to ESI-MS analysis (Fig. 4). Over 20 oligonucleotides with unique sequences and lengths were analyzed. Table 2 shows the mass accuracy and amount injected for each synthetic oligonucleotide. Average mass accuracy for all LC/MS experiments was 80 ppm. This means that a 30 mer mass measured (10 000 Da) is usually within ± 0.8 Da of the theoretical value. As little as 100 picomoles (pmol) of synthetic oligonucleotide was used for analysis, which afforded excellent signal-to-noise (S/N) ratios. Based on a S/N ratio of 5:1 in the deconvoluted spectrum, the calculated limit of detection (LOD) for the 25 mer (Table 2) is ~2 pmol. For 100 mers the LOD was calculated to be ~140 pmol. The actual LOD of the method is probably lower, due to the fact that oligonucleotide synthesis yield is not 100%. Calculations for LOD were made with the assumption that all samples were 100% pure, which is a conservative estimate.

The on-line LC/MS method was compared with MALDI-TOFMS for synthetic oligonucleotide QC. Figure 5 shows the spectra for 100 pmol of a synthetic 40 mer oligonucleotide analyzed by LC/MS (Fig. 5(A)) and MALDI-MS (Fig. 5(B)). It appears that the resolution and LOD of the LC/MS method are better suited for oligonucleotides longer than 35 mer. Samples were desalted prior to MALDI-MS analysis using previously published protocols.[11,13]

Application to SNP genotyping

Since the rapid on-line HPLC desalting method was found to be suitable for the routine MS analysis of synthetic oligonucleotides, it was then applied to the area of single nucleotide polymorphism (SNP) genotyping. The method utilized in this research to generate SNP-laden oligonucleotides is described in detail in the Experimental section. Briefly, a region of genomic DNA containing a SNP of interest is

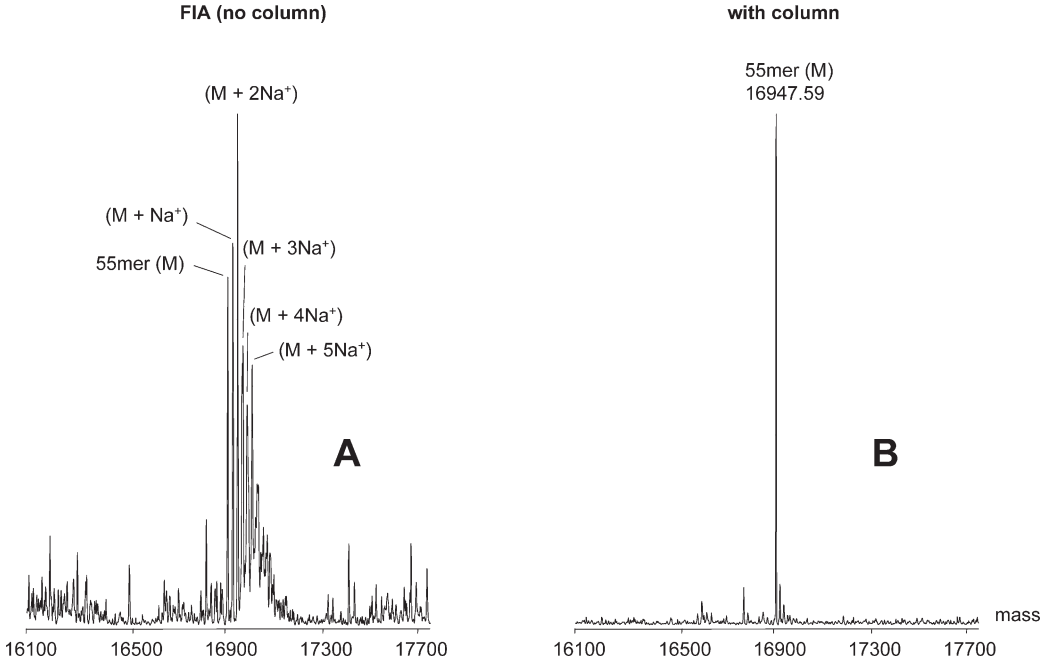

Figure 3. Flow injection (A) and rapid LC/MS (B) analysis of a 55 mer heterooligonucleotide. Both spectra shown are deconvoluted from multiply charged raw spectra. For FIA experiments, samples were injected onto the system shown in Fig. 2 without a column present. Rapid LC/MS was performed according to method 3 in Table 1. Oligonucleotide sequence specified in Experimental section.

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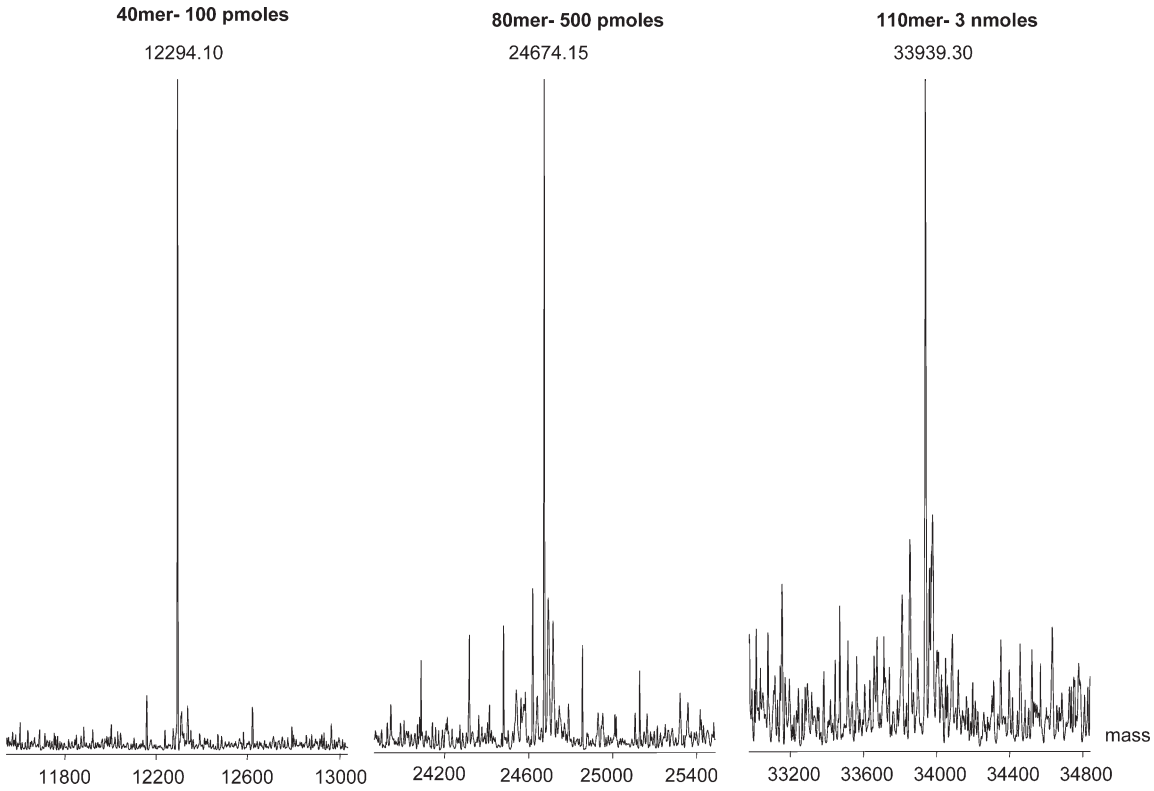

Figure 4. Deconvoluted ESI-TOF mass spectra of 40–110 mer synthetic heterooligonucleotides desalted by rapid LC/MS. Desalting prior to ESI-TOFMS analysis was performed using method 3 in Table 1 and the instrumental setup shown in Fig. 2. The mass accuracy values for the oligonucleotides shown can be found in Table 2. Oligonucleotide sequences specified in Experimental section.

amplified by PCR. This is followed by a linear amplification of the SNP-containing region through the use of a nicking enzyme, which recognizes a 5-base site inserted into each primer. As a result, short single-stranded DNA fragments (8–16 mer) are formed, which can then be easily analyzed by many analytical methods, including ESI-TOFMS. Homozygotes for a given SNP produce two single DNA strands, corresponding to the forward and reverse strands of the double-stranded DNA template. Similarly, heterozygotes will produce four single DNA strands as a result of the nicking reaction.[30] The challenge is to adequately desalt the SNP fragments after enzymatic reactions, which are contaminated with PCR reaction components and buffer constituents. These contaminants include enzymes, glycerol, surfactants, 50–100 mM alkali salts, and many other components.

The rapid LC/MS method was found to be suitable for efficient desalting of SNP samples generated using the method described above. Figure 6 shows the deconvoluted spectra of two representative SNP genotyping samples that were desalted with the optimized LC/MS method. The individual in Fig. 6(A) is homozygous for the C allele of the HGBASE-10041 SNP (Table 3). The subject in Fig. 6(B) is heterozygous for the A and T alleles of the IL8-353 SNP (Table 3). Notice that the resolution of the ESI-TOF instrument is sufficient to differentiate the A and T strands (difference of 9.0 Da). This is the smallest expected mass difference between SNP samples, and is consequently the most challenging to resolve. It is also interesting that the method detects non-templated adenine base additions to the DNA primer (Fig. 6). These are common when using *Taq* DNA polymerases for PCR amplification. While adenine extension of the primer may potentially complicate genotyping, the correct genotype call can be made in such cases due to the use of MS for analysis.

Table 2. Rapid LC/MS analysis of 25–110 mer synthetic oligonucleotides

Length	Mass load (nmol)	Theoretical MW (Da)	Experimental MW (Da)	Difference (Da)	Mass accuracy (ppm)
25 mer	0.1	7680.07	7679.33	−0.74	96.0
35 mer	0.1	10 744.06	10 744.69	0.63	58.6
40 mer	0.1	12 293.07	12 294.15	1.08	87.9
55 mer	0.1	16 946.10	16 947.63	1.53	90.5
70 mer	0.1	21 559.10	21 559.52	0.42	19.5
80 mer	0.5	24 672.13	24 674.93	2.80	113.5
90 mer	1.0	27 736.12	27 738.35	2.23	80.3
100 mer	1.0	30 825.13	30 827.15	2.02	65.6
110 mer	3.0	33 938.16	33 939.85	1.69	49.8
			Average	**1.30**	**73.5**

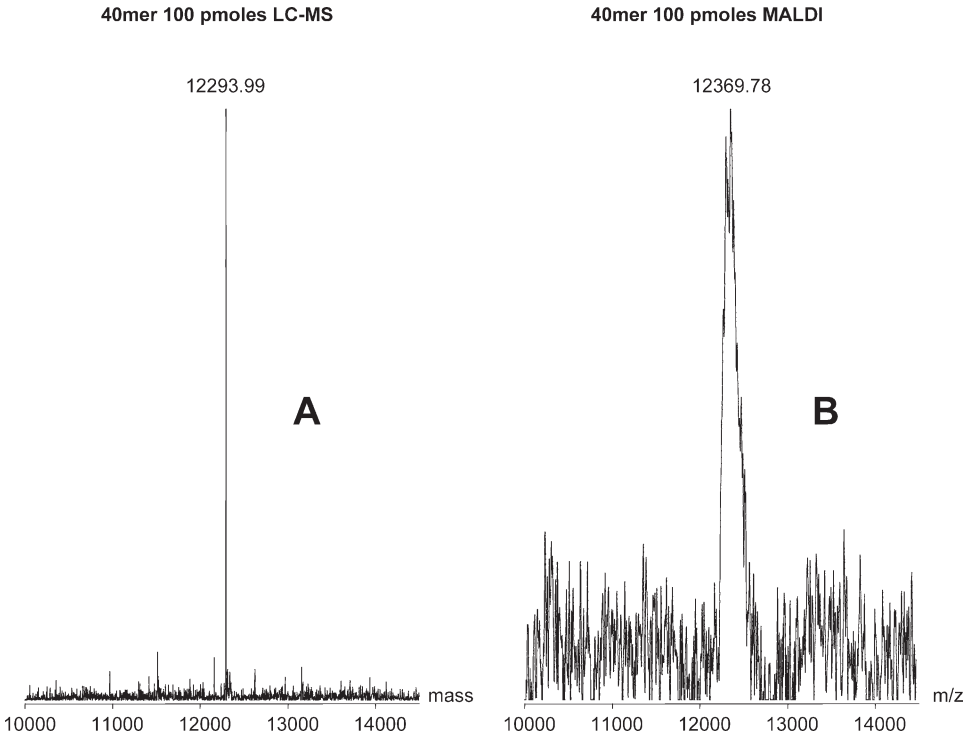

Figure 5. Analysis of a 40 mer heterooligonucleotide by rapid LC/MS (A) and MALDI-TOFMS (B). Rapid LC/MS performed according to method 3 in Table 1. Setup identical to that in Fig. 2. MALDI parameters specified in the Experimental section.

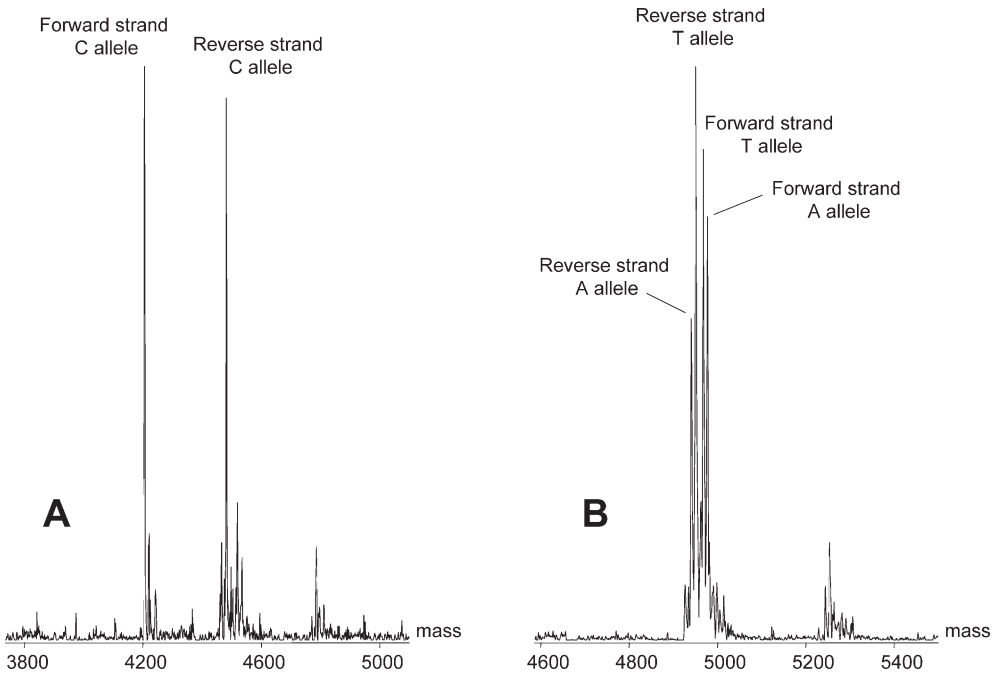

Figure 6. Deconvoluted ESI-TOF mass spectra of an individual who is homozygous for the C allele of the HGBASE-10041 SNP (A) and an individual who is heterozygous for the A and T alleles of the IL8-353 SNP (B). Samples were desalted using method 3 in Table 1 and the setup in Fig. 2. The peaks present at about 4800 Da in (A) and 5250 Da in (B) are non-templated adenine base additions to the forward and reverse strands of the alleles. This is common when using *Taq* DNA polymerases in the PCR step.

The on-line desalting method was utilized for a total of 180 SNP genotyping reactions. Genotyping experiments were performed with six different SNPs and three different human genomic DNA samples. Results from these experiments are shown in Table 3. With the exception of three failed analyses, the correct genotyping call was made 100% of the time. Ambiguous calls or no calls were caused by HPLC instrument malfunction, or by PCR or nicking reaction failures

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Table 3. Rapid LC/MS analysis results for SNP genotyping experiments

		Human DNA NA15029[a]		Human DNA NA15221[a]		Human DNA NA15506[a]	
SNP label	Variants	Genotype	Frequency (%) (10 wells total)	Genotype	Frequency (%) (10 wells total)	Genotype	Frequency (%) (10 wells total)
PG58320	G,A	Homozygous A	90[b]	Homozygous A	100	Heterozygous G/A	90[c]
HGBASE-10041	C,T	Homozygous C	100	Heterozygous C/T	90[b]	Homozygous C	100
BDNF-V66M	G,A	Homozygous G	100	Heterozygous G/A	100	Homozygous G	100
NAT1-S214A	G,T	Homozygous T	100	Homozygous T	100	Homozygous T	100
IL1B-3954	C,T	Homozygous C	100	Homozygous C	100	Homozygous C	100
IL8-353	T,A	Heterozygous A/T	100	Homozygous T	100	Homozygous T	100

[a] Individual human genomic DNA was purchased from the Coriell Institute (Camden, NJ, USA).
[b] Sample was lost due to HPLC system malfunction during injection.
[c] PCR or nicking reaction failure.

(Table 3). Mass accuracy obtained for all SNP genotyping experiments was comparable to that obtained with synthetic oligonucleotides (<100 ppm).

Analysis of large sets of genotyping samples revealed that many of the PCR contaminants (surfactants, enzymes, etc.) were building up on the HPLC column over time. However, a short (~5–7 min) regeneration at 75% ACN was sufficient to restore the column to its original condition. This regeneration was performed every 96 samples. The column was used for ~1000 injections of synthetic oligonucleotides and PCR reactions without noticeable deterioration in desalting performance.

The results clearly demonstrate the utility of the desalting method for complicated PCR samples prior to ESI-MS analysis for the identification of DNA genotypes. Further, due to the current throughput needs of many genotyping laboratories (>1000 samples/day), the rapid LC/MS method described here could be routinely implemented for that purpose.

CONCLUSIONS

A fast on-line desalting LC/MS method was successfully applied for the high-throughput quality control of synthetic oligonucleotides. With a duty cycle time of 1.5 min/sample (including injection, loading/washing, elution, and equilibration), approximately 950 samples per 24-h time period can be analyzed. The method was found to be highly efficient for removing alkali cation contaminants from nucleic acids prior to ESI-MS analysis. The method is also suitable for analyzing long oligonucleotides (>50 mer) that are difficult to analyze by MALDI-TOFMS. Average mass accuracy for the LC/MS method is 80 ppm, with LOD values in the range 2–140 pmol (25–100 mer). The high-throughput desalting method was also found to be efficient for desalting SNP genotyping samples generated by PCR prior to ESI-TOFMS analysis.

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