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Sample Self-Displacement in the Mass-Directed Purification of Synthetic Peptides and Oligonucleotides

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Overview

- Synthetic peptides were purified on a commercially available system using automated mass spectrometry directed fractionation.
- The sample self-displacement mechanism was investigated using electrospray ionization mass spectrometry (ESI-MS) under overloading conditions on analytical sized columns.
- Up to 20 mg of synthetic peptide was purified in a single injection on a 4.6 mm i.d. column with high yield and purity.
- Re-injection of the collected peptides onto the LC-MS system routinely gave greater than 98% purity.

Introduction

Mass spectrometry (MS) directed fraction collection was utilized in conjunction with the sample self-displacement mode of separation for automated purification of synthetic peptides and oligonucleotides. In the sample self-displacement mode, weakly retained compounds are displaced from the chromatographic sorbent by more strongly retained compounds. As the injected mass load increases, the analytes start to form a displacement train, eluting as "square wave" zones rather than peaks (Fig.1). Using this method, it is possible to purify large amounts of peptides on analytical columns (Table 1).

Separation of a 25mer phosphorothioate (PS) oligonucleotide at increasing mass loads demonstrates the efficiency of the sample self-displacement mechanism (Fig. 2). A similar displacement mechanism was confirmed for synthetic peptides on a 2.1 × 150 mm, 5 µm, C_{18} column (Fig. 3). These experiments prove that the more hydrophobic sample components are displacing the more hydrophilic ones, thus maintaining the displacement train on-column, and achieving adequate resolution for neighboring analytes. In this manner, up to 20 mg of synthetic peptide was injected and purified using automated MS-based fraction collection on a 4.6 mm i.d. column (Fig. 4) without reaching the limit of the self-displacement separation mode. Re-injection of the collected peptide(s) onto the LC-MS system routinely indicated greater than 98 % purity.

Experimental

HPLC: Capillary separations were performed on a CapLC® Separations Module equipped with a 2996 photodiode array (PDA) detector (Waters Corporation, Milford, MA,USA). Analytical separations were performed on an Alliance® Bioseparations system equipped with a 2996 PDA detector (Waters). Finally, peptides were purified in automated fashion on a Waters FractionLynx™ MS-based Autopurification system equipped with a 2996 PDA detector and Micromass® ZQ™ single quadrupole mass spectrometer. All HPLC and MS instruments were controlled by the manufacturer's software. For HPLC conditions, see figure captions.

MS: A Micromass® LCT™ (Waters) was used for analysis of oligonucleotides eluting from the CapLC® system. The Micromass® ZQ™ was used for detection of peptides eluting from the Alliance® Bioseparations system and FractionLynx™ MS-based Autopurification system. For MS conditions, see figure captions.

Columns: XTerra® MS C_{18}, 1.0 × 50 mm, 2.5 µm (oligonucleotides); Atlantis™ dC_{18} 2.1 × 150 mm, 5 µm (peptides), Atlantis™ dC_{18} 4.6 x 100 mm, 5 µm (preparative peptide purifications). All columns manufactured by Waters Corporation.

Solvents: For oligonucleotides, mobile phase A consisted of 100 mM hexafluoroisopropanol (HFIP) and 8.6 mM triethylamine (TEA), pH 8.33. Mobile phase B was 100 % MeOH. For peptides, mobile phase A was comprised of 0.1% trifluoroacetic acid (TFA) in water, and mobile phase B consisted of 0.08% TFA in acetonitrile (ACN).

Samples: The synthetic 25mer PS oligonucleotide was supplied by Hybridon, Inc. (Cambridge, MA, USA). Synthetic peptides were provided by either Cell Essentials (Boston, MA, USA) or SynPep (Dublin, CA, USA).

Results and Discussion

Figure 1: Sample self displacement mechanism using gradient chromatography. Weakly retained compounds (impurities B, and C) are displaced from the chromatographic sorbent by more strongly retained compounds (target peptide, impurity A). When the column is heavily overloaded, a displacement train develops, and analytes elute as "square wave" zones rather than peaks. These analytes are eluted from the column by a gradient of organic displacer (i.e., ACN or MeOH).

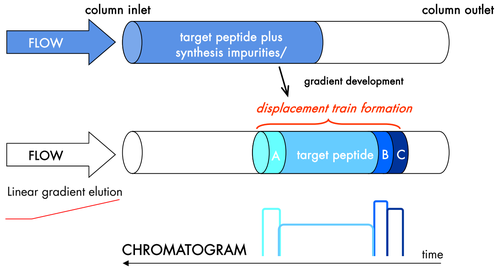
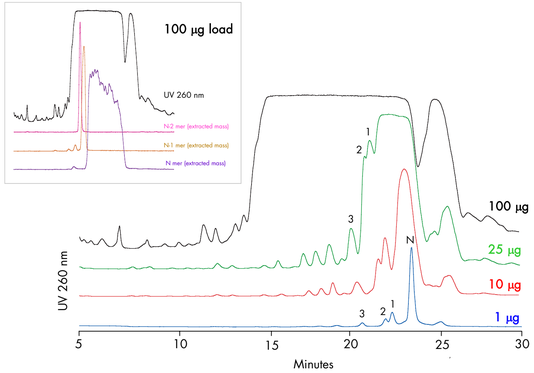
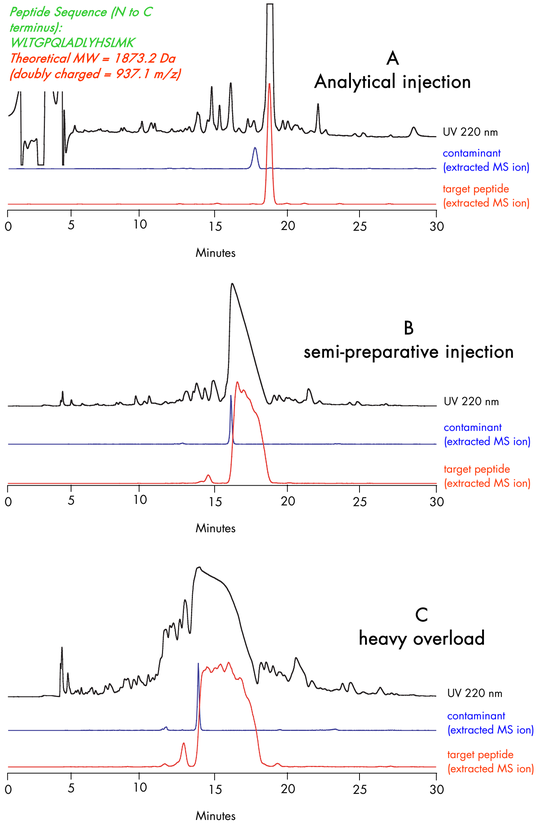


Figure 2: UV detection of a 25mer PS (N) becomes saturated at high mass loads. At the 100 µg load (1.0 mm i.d. column), it is unclear what the collection window for purification should be. The ESI-MS extracted ion chromatograms (figure inset) indicate that even though the UV detector is saturated, the target product (Nmer) is still well separated from major synthesis impurities (Nmer-1, Nmer-2).



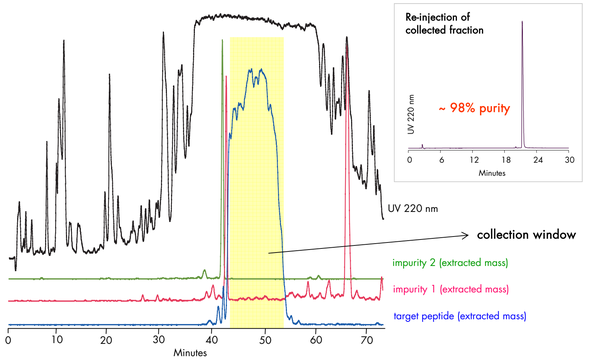
Conditions: CapLC® Separations Module equipped with a 996 PDA detector and Micromass® LCT™ mass spectrometer. XTerra® MS C_{18}, 1.0 x 50 mm, 2.5 µm column. Mobile phase A: 100 mM HFIP: 8.6 mM TEA, pH 8.33; B: MeOH. Linear gradient from 0—13% B in 1 minute, followed by a linear gradient from 13—20% B in 28 minutes; 60 °C; 23.6 µL/min.; UV 260 nm. ES- mode; capillary 2.5 kV; cone 35 V; extractor 1 V; MCP detector 2700 V; source temperature 100 °C; desolvation temperature 120 °C; cone gas 50 L/hr.; desolvation gas 430 L/hr. Scan from 340—1500 m/z; scan time 1.0 s; 0.1 s delay.

Figure 3: Injection of 2 µg (A), 200 µg (B), and 1 mg (C) of synthetic peptide onto a 2.1 mm i.d. column. Proof of sample self-displacement can be seen in the decreasing retention times of the target peptide (m/z = 1873.2 Da) and one of the closely-eluting synthesis contaminants (m/z = 758.1 Da) with an increase in mass injected on-column.



Conditions: Alliance® Bioseparations system equipped with a 2996 PDA detector and Micromass® ZQ™ mass spectrometer. Atlantis™ dC_{18}, 2.1 x 150 mm, 5 µm column. Mobile phase A: 0.1% TFA in water; B: 0.08% TFA in ACN. Linear gradient from 18—42% B in 30 minutes; ambient temperature; 0.2 mL/min.; UV 220 nm. ES+ mode; capillary 3.2 kV; cone 30 V; extractor 3 V; source temperature 100 °C; desolvation temperature 350 °C; cone gas 155 L/hr.; desolvation gas 360 L/hr. Scan from 300—2500 m/z; scan time 2.2 s; 0.1 s delay.

Figure 4: Automated MS-directed purification of 20 mg of synthetic peptide on a 4.6 mm i.d. column (same peptide as Fig. 3). Fraction collection was triggered by selected ion monitoring (target peptide MW = 1874.0). The figure inset is an analytical injection of the collected fraction, which has greater than 98% purity (initial purity was ~ 86%).



Conditions: Waters FractionLynx™ MS-based Autopurification system equipped with a 2996 PDA detector and Micromass® ZQ™. Atlantis™ dC_{18}, 4.6 x 100 mm, 5 µm column. Mobile phase A: 0.1% TFA in water; B: 0.08% TFA in ACN. Initial hold at 10% B for 1 minute, followed by a linear gradient from 10—50% B in 90 minutes; ambient temperature; 1.0 mL/min.; UV 220 nm. ES+ mode; capillary 3.5 kV; cone 25 V; extractor 3 V; source temperature 150 °C; desolvation temperature 250 °C; cone gas 50 L/hr.; desolvation gas 260 L/hr. Scan from 320—1920 m/z; scan time 2.2 s; 0.1 s delay.

Table 1: Comparison of scale-up calculations for preparative peptide purification using traditional elution chromatography and MS-directed purification via sample self-displacement (constant column length = 100 mm). Calculations were based on a 20-fold increase in mass load capacity with sample self-displacement.

Column i.d. (mm)	Mass Load Traditional Elution Chromatography	Mass Load Sample Self-Displacement	Flow rate (mL/min.)
4.6	1 mg	20 mg	1
10.0	5 mg	100 mg	5
19.0	20 mg	340 mg	17
30.0	45 mg	0.85 g	43
50.0	120 mg	2.4 g	118

Conclusions

- A method was developed for the automated purification of oligonucleotides and peptides using MS-directed fraction collection on a commercially available system.
- The sample self-displacement mechanism was demonstrated on narrowbore and analytical size columns.
- Sample self-displacement in combination with MS-directed fraction collection allowed preparative amounts of synthetic peptide to be purified on 4.6 mm i.d. columns.
- Sample purity was routinely above 98%.
- Up to 20-fold more sample can be purified using this method compared to traditional elution chromatography.