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Comparison of ESI-MS interfaces for the analysis of UV-crosslinked peptide–nucleic acid complexes

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Abstract

In this report, the effectiveness of high performance liquid chromatography (HPLC) in conjunction with electrospray ionization mass spectrometry (ESI-MS) is examined as a tool for identifying the sites of crosslinking in a protein that has been photoreacted with a non-photolabeled oligonucleotide. ESI-MS and MALDI-MS analyses preceded by off-line microflow and nanoflow HPLC, on-line microflow HPLC/ESI, and on-line nanoflow HPLC/ESI interfaces were performed in order to determine their relative effectiveness in separating mixtures of nucleopeptides and identifying sites of crosslinking on the individual components. The characteristics of these four techniques as well as possibilities for improving the analysis of nucleopeptides by ESI-MS are compared and discussed.

Keywords: Nanoscale liquid chromatography; Electrospray ionization mass spectrometry; UV-crosslinking; Peptide–nucleic acid complexes

1. Introduction

The use of photochemical crosslinking as a tool for locating the sites of interaction between proteins and nucleic acids dates back to the early 1960s [1]. The UV light fixes the interaction between a protein and a nucleotide by inducing a "zero-length", covalent bond between the two biopolymers [2]. Although photochemical crosslinking for the formation of the covalent bonds is not fully understood, the technique has been exploited to investigate both static and dynamic protein–nucleic acid complexes [3,4]. In the 1990s, Barofsky and coworkers demonstrated that matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) could be effectively employed to identify the DNA-binding domains in proteins photochemically crosslinked to oligonucleotides [5,6]. This strategy of combining photochemical crosslinking and mass spectrometry has been used by several investigators to characterize the interface between protein and oligonucleotide in a variety of heteroconjugates. This body of work, as well as the various strategies that have emerged to photochemically produce and mass spectrometrically analyze protein/peptide–nucleic acid heteroconjugates, was comprehensively reviewed by Steen and Jensen [7].

The potential for sequencing peptide–nucleic acid complexes via electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was first demonstrated using a synthetic peptide chemically linked to the oligonucleotide dT_6 [8]. Results from this early study showed that ESI-MS/MS could sequence the peptide portion of the complex, sequence the oligonucleotide portion of the complex, and identify the chemically crosslinked amino acid. Apparently, the first application of ESI-MS/MS to the analysis of a peptide–oligonucleotide heteroconjugate derived from a photochemically crosslinked protein–DNA complex was made in 1999 [9]. Subsequently, other laboratories have used it to characterize the interface between peptide and oligonucleotide in

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heteroconjugates formed either by photochemical crosslinking [10-12] or by chemical crosslinking [13,14]. Interestingly, in those mass spectrometric studies of photochemical crosslinking where the nucleic acid substrate was photolabeled (usually with either 5-bromo- or 5-iodouracil) [12,15], crosslinking to the protein was found to occur only at a single amino acid residue regardless of the method of mass analysis. In those studies where the nucleic acid substrate was not photolabeled, crosslinking to the protein was found to occur at a number of amino acid sites [10,11].

In the present study, complex mixtures of dT_n ($n = 2$–6) nucleopeptides were isolated and characterized by microflow/nanoflow chromatography coupled off-line or on-line with ESI and MALDI mass spectrometry. The goal of this survey was to find which chromatographic configuration allowed for a complete mass spectrometric characterization of the nucleopeptide mixture.

2. Experimental

2.1. Chemicals

Acetic acid (AA), trifluoroacetic acid (TFA), formic acid (FA), diammonium hydrogen citrate, tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCL), and 2,4,6-trihydroxyacetophenone (THAP) were purchased from Sigma Chemical Co. (St Louis, MO). HPLC grade acetonitrile, ammonium acetate, ammonium bicarbonate, glycerol, dithiothreitol, sodium chloride, ethylenediaminetetraacetic acid (EDTA), isopropanol and methanol were supplied by Fisher Scientific (Pittsburgh, PA). Water was generated with a Milli-Q water purification system (Millipore Corp., Bedford, MA). Oligodeoxythymidylate dT_{20} was synthesized by the Biopolymer Core Facility, at the University of Maryland at Baltimore and further purified by native polyacrylamide (15%) gel electrophoresis. Ung was overexpressed and purified as described by Bennett et al. [16] with modifications described by Sanderson and Mosbaugh [17].

2.2. UV crosslinking

Mixtures of crosslinked nucleopeptides were produced by placing 36 nmol of purified Ung and a 3-fold molar excess of dT_{20} in a quartz cuvette (4 mL) and adding DAB buffer (30 mM Tris–HCl, 50 mM NaCl, 1 mM dithiothreitol, 5% (w/v) glycerol, pH 7.4) to bring the final volume to 1 mL; placing the cuvette on ice for 15 min, and finally, laying the cuvette lengthwise on a bed of ice in a Stratalinker 1800 (Stratagene Cloning Systems, La Jolla, CA) and irradiating ($\lambda_{max} = 254$ nm) the reaction mixture for 15 min. The irradiated solution was either used immediately or stored at −80 °C.

2.3. Isolation of nucleopeptide mixtures

The Ung–oligonucleotide complexes produced by photocrosslinking were isolated using denaturing polyacrylamide gel electrophoresis (PAGE) and digested in the gel with trypsin (36 ng/μL) as described by Shevchenko et al. [18]. The peptide × dT_{20} fragments (nucleopeptides) resulting from this procedure were extracted and purified as described in detail by Gafken [19]. Briefly, the aqueous digestion solution was removed and saved. The tryptic peptides remaining in the gel were extracted in two stages, respectively, into FAPH (50% formic acid, 25% acetonitrile, 15% isopropyl alcohol, and 10% water) and acetonitrile. The FAPH and acetonitrile were combined, concentrated to dryness by vacuum centrifugation, and re-suspended with 250 μL of 10 mM Tris–HCl (pH 7.2), 1 mM EDTA, and 100 mM sodium chloride (TE-100 buffer). The aqueous digestion solution saved earlier was diluted in TE-100 buffer and mixed with the resuspended organic extract. This composite solution of tryptic proteolysis products was loaded onto a NAC-52 anion exchange cartridge (Life Technologies Inc., Grand Island, NY); the cartridge was washed with 5 mL of TE-100; and the nucleopeptides were eluted with 1 mL of TE-1000 (10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 1000 mM NaCl) directly into a Centricon-3 (Millipore, Billerica, MA) cartridge for centrifugal desalting. The washed sample was concentrated to 200 μL and then transferred to a 750-μL Eppendorff tube and further concentrated to about 10 μL by vacuum centrifugation. Nuclease P1 (Amersham Biosciences, Piscataway, NJ) was diluted with 50 mM ammonium acetate to 0.04 activity units/μL. The isolated peptide × dT_{20} complexes were digested by mixing 1 μL of nuclease P1 solution with 1 μL of nucleopeptide sample and allowing the reaction to proceed at 37 °C for 4 h.

2.4. Off-line microflow HPLC

Chromatography was performed off-line on a system made up of an ABI 140B syringe pump (Applied Biosystems, Foster City, CA), a flow splitter (Upchurch Scientific, Murrieta, CA), a 0.32 mm (i.d.) × 250 mm column packed in-house with 5 μm, 150 Å pore, BetaBasic C_{18} packing material (Keystone Scientific/Thermo Electron Corporation, San Jose, CA), and an ABI 759 UV detector (Applied Biosystems, Foster City, CA) equipped with a 35-nL dead volume, 8-mm path-length flowcell (LC Packings, Sunnyvale, CA). All connections in the system were made with 50 μm (i.d.) silica tubing (Polymicro Technologies, Phoenix, AZ). The solvents used in the mobile phase were 5 mM ammonium acetate (A) and 100% acetonitrile (B); solvent was delivered at 5 μL/min while linearly increasing the concentration of B from 5% to 50% over an interval of 45 min. Fractions containing species detected at 217 nm were collected off-line for subsequent identification by MALDI and nanospray MS.

2.5. Matrix-assisted laser-desorption ionization MS

MALDI MS was performed on a custom-built, delayed extraction, time-of-flight instrument [5]. The matrix solution containing 50% acetonitrile and 50 mM diammonium hydrogen citrate was saturated with 2′,4′,6′-trihydroxyacetophenone (THAP). Nucleopeptide sample and matrix were mixed in a ratio of 1:2 (v:v), and 1 μL of this mixture was applied to a pre-crystallized layer of THAP on a stainless steel probe. After allowing the spot to dry, it was rinsed with deionized water

and again allowed to dry. The ion source potential was set at +20 kV, and the extraction delay was set at 500 ns. Mass spectra were produced using *MoverZ* software (Genomic Solutions Inc, http://www.genomicsolutions.com) to sum the signals generated from 30 individual laser pulses.

2.6. Nanoelectrospray (nano-ESI) MS

The nanospray analyses were performed on an API III triple quadrupole mass spectrometer (MDS Sciex, Concord, Ontario) in which the commercial electrospray source was replaced with a nanospray source constructed by the Protein and Peptide Group in the European Molecular Biology Laboratory (Heidelberg, Germany) [20]. Nanospray needles were purchased from Protana Inc. (Toronto, Ontario, Canada). The flow rate of the nitrogen curtain gas was 0.6 L/min; the needle voltage was 700 V; the interface plate voltage was 100 V; and the orifice potential was 80 V. Each sample collected off the HPLC system was adjusted to 1% formic acid and 40% acetonitrile; 0.6 μL of adjusted sample was loaded into a nanospray needle. MS spectra were acquired by stepping the first quadrupole in *m/z*-increments of 0.3 over 3 s scan intervals.

2.7. On-line microflow HPLC/ESI-MS

The chromatography system comprised an ABI 140B syringe pump (Applied Biosystems, Foster City, CA), a flow splitter (Upchurch Scientific, Murrieta, CA), and a 0.17 mm (i.d.) × 100 mm column packed in-house with 5-μm, 100-Å pore, Luna C_{18} packing material (Phenomenex, Torrance, CA). The solvents used in the mobile phase were 0.1% acetic acid, 0.01% trifluoroacetic acid (A) and 0.1% acetic acid, 0.01% trifluoroacetic acid in acetonitrile (B); solvent was delivered at 2 μL/min while linearly increasing the concentration of B from 5% to 50% B over an interval of 30 min. The HPLC column was connected by 30 μm (i.d.) silica tubing to the custom-built ESI source of a Finnigan LCQ ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA). The ESI sprayer was a 2.5 cm long, 35 gauge, stainless steel hypodermic needle mounted on an XY-manipulator; the tip of the needle was positioned approximately 5 mm from the mass spectrometer's inlet capillary. During operation, the spray-needle was held at 2.3 kV, the temperature of the capillary inlet was maintained at 180 °C, the capillary potential at 46 V, and the tube-lens offset-potential at 30 V. Mass spectra were acquired between *m/z* 400 and 2000 with a maximum injection time set at 50 ms.

2.8. Off-line nano-LC/ESI-MS

A nano-LC column was prepared by packing a 15 cm length of 360 μm (o.d.) × 75 μm (i.d.) fused-silica (Polymicro Technology, Phoenix, AZ) with 5 μm, 300 Å pore, Luna C_{18} silica gel particles (Phenomenex, Torrance, CA) using a pressurized bomb method described by Kennedy and Jorgensen [21]. In short, the end of the silica capillary that is to become the inlet was slipped through a seal into the bomb containing a slurry of isopropanol and the packing material (25 mg/mL); the outlet of the column was inserted into a Valco (Houston, TX) microbore end-fitting containing a 2 μm Valco metallic screen that acted as a temporary outlet frit. The packing material was forced into the capillary by pressurizing the bomb to 1500 psi; about 2 h were required to pack a 15 cm long column. After the gas pressure was slowly released from the bomb, the inlet of the column was connected to an HPLC pump and flushed with acetonitrile for 2 h and Milli-Q water for another 2 h. A fused-silica splicer (Fujikura, Japan) was used to prepare the final outlet frit while the column was still wet. An exponential dilution method described in detail elsewhere [22] was used to produce gradient separations. Briefly, a ZDV PEEK union (Upchurch Scientific, Murrieta, CA) drilled out to an internal volume of 3 μL and the 10 μL inlet port of the micro-injection valve (Rheodyne Model 8125, Rohnert Park, CA) were used as the mixing chambers. Two Kratos Spectroflow 400 HPLC pumps (Kratos Analytical, Chestnut Ridge, NY) were used to independently deliver solvent A (0.1% AA plus 0.01% TFA) and solvent B (0.1% AA plus 0.01% TFA in 50% acetonitrile) to the mixing chambers. PEEK tubing (Upchurch Scientific, Murrieta, CA) with 60 μm i.d. was used to connect the pumps to the filter, splitter, mixing chamber, and injector. The inlet of nanocolumn was attached directly into the injector's outlet port. The outlet of the nanocolumn was connected via a 30 μm (i.d.) × 20 cm fused-silica transfer line to a capillary electrophoresis (CE) unit (Beckmann P/ACE System 2210, Fullerton, CA) which provided UV detection and data acquisition. The nanocolumn was attached to the transfer capillary with a short piece of Teflon tubing; the length of the transfer capillary from the outlet of the nanocolumn up to the detector was 13 cm. A small diameter hole was drilled in the upper part the CE unit's cartridge (the housing that holds the capillary in place) to allow the packed capillary column to be connected to the transfer line inside the cartridge. A small hole, aligned with the hole in the cartridge, was also drilled in the upper right part of the cartridge interface of the CE unit.

Sample was displaced from the injection valve's loop (5 μL) onto the nanocolumn by forcing solvent A through the system at 0.8 μL/min (inlet pressure = 200 bar) for ∼15 min, and it was separated on the column with an exponential gradient produced by forcing solvent B through the system at 0.3 μL/min (inlet pressure = 120 bar). Fractions were collected manually for subsequent analyses by MALDI and nanoelectrospray MS. This was performed by adding 1 μL of solvent A to a vial and collecting 2 μL of eluate over a 2-min period to bring the final volume in the vial to 3 μL.

2.9. On line nano-LC/ESI-MS

Analyses were performed on the LCQ ion trap mass spectrometer operated with the same experimental parameters described in a preceding subsection. A 360 μm (o.d.) × 75 μm (i.d.) × 40 cm fused-silica capillary with a 15 μm tip and an integral frit (New Objective "PicoFrit", Cambridge, MA) was packed with 5 μm, 300 Å pore, Luna C_{18} particles. The column was connected directly to the 8125 Rheodyne injector placed in a plastic box, and the spraying voltage (2.5 kV) was applied

to the injector after loading the sample. Elution gradients were produced using the exponential dilution setup described in the preceding subsection by mixing solvent A (0.1% AA plus 0.01% TFA) and solvent B (0.1% AA plus 0.01% TFA in 40% acetonitrile).

3. Results

3.1. Off-line microflow HPLC/MALDI MS

Prior to analysis by ESI-MS, an aliquot of the nuclease P1 digest of the tryptic nucleopeptides was surveyed by MALDI MS to gauge the separation efficiency. The resulting mass spectra revealed that the sample contained a number of complexes of the form peptide × dT_n ($n = 2$–6), i.e. oligodeoxyribothymidylic acids of various lengths crosslinked to certain tryptic peptides (data not shown). Separation of the peptide–nucleic acid mixture by microflow-HPLC produced two late-eluting fractions (Fig. 1A). The MALDI mass spectrum of Fraction I (Fig. 1B) shows two series of singly charged ion species. The members of the dominant series (*m/z* 1796.0, 2100.2, 2404.4, 2708.6, and 3012.8) correspond, respectively, to the polydeoxyribothymidylic acids dT_2 through dT_6 crosslinked with the Ung–peptide T18 [184-APHPSPLSAHR-194]. The signals in the weaker series (*m/z* 2020.1, 2324.3, 2628.4, and 2932.7) correspond, respectively, to dT_3 minus a phosphate through dT_6 minus a phosphate crosslinked with T18. A signal due to the free peptide T18 itself also appears in the spectrum at *m/z* 1169.6.

The MALDI mass spectrum of Fraction II (Fig. 1C) shows nucleopeptide ion signals at *m/z* 2315.3, 2485.5, and 2703.8 corresponding to dT_2 crosslinked, respectively, with $T6_{(-7)}$, $T6_{(-5)}$, and $T6_{(-3)}$ (where these three peptides are hydrolysis products of T6 [57-VVILGQDPYHGPGQAHGLAFSVR-79] from which seven, five, and three C-terminal amino acids, respectively, have been lost during sample preparation), at *m/z* 3312.2 corresponding to dT_4 crosslinked with $T6_{(-3)}$, and at *m/z* 2570.5, 2874.7, and 3178.9 corresponding, respectively, to dT_2, dT_3, and dT_4 crosslinked with T11 [129-AGQAHSHASLGWETFTDK-146].

3.2. Off-line microflow HPLC/nano-ESI-MS

Nanospray analyses of the Fractions I and II (Fig. 2) produced results similar to those obtained by MALDI MS. The Q1 mass spectrum of Fraction I (Fig. 2A) exhibits mass peaks at *m/z* 700.7, 802.1, 903.5, and 1004.9 corresponding to triply charged T18 × dT_n with *n* equals to 3 through 6, respectively. Another series of triply charged T18 × dT_n ions ($n = 4$–6) in which the nucleotide has lost a phosphate group is seen in the spectrum at *m/z* 775.4, 876.8, and 978.2, respectively. The nanospray Q1 mass spectrum of Fraction II (Fig. 2B) contains mass peaks corresponding to $T6_{(-7)}$ × dT_2, $T6_{(-5)}$ × dT_2, T11 × dT_3, and T11 × dT_4 (*m/z* 772.4, 829.2, 958.9, and 1060.3, respectively). The mass spectra from both HPLC fractions indicate that significant cation adduction occurred in the nanospray process. By contrast, no evidence of cation adduction was found in the MALDI mass spectra.

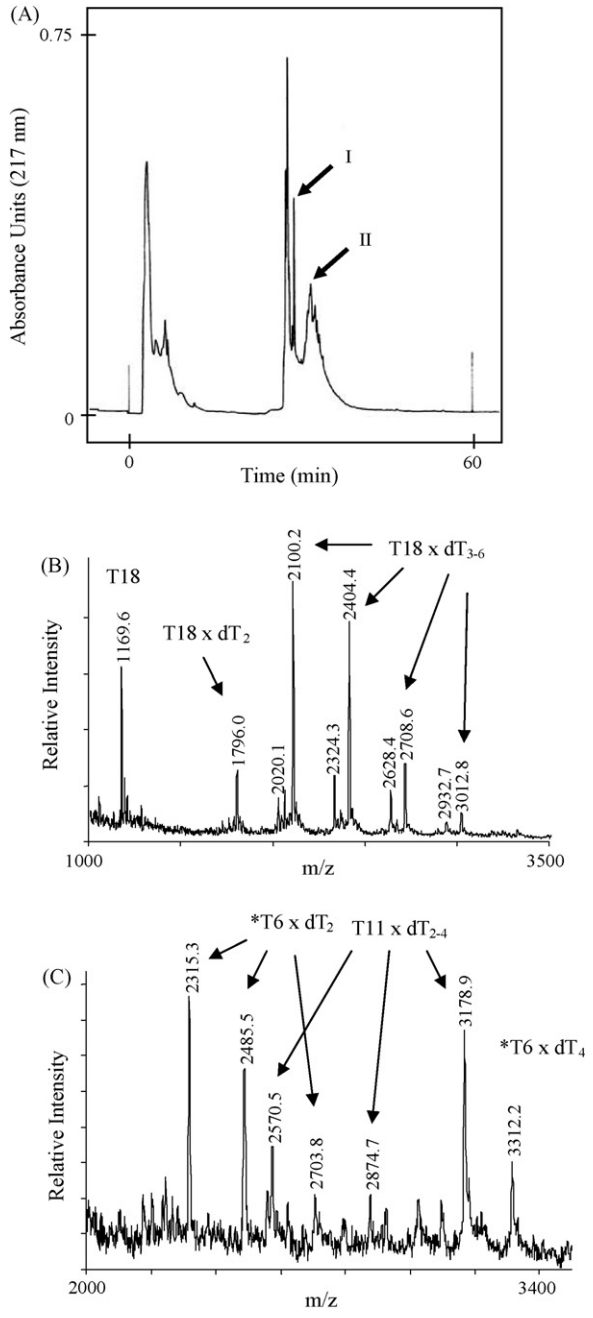

Fig. 1. Off-line HPLC isolation of nucleopeptides. The LC/UV trace of a nuclease P1 digest of tryptic nucleopeptides indicating the fractions labeled I and II (A). MALDI spectra of Fraction I (B) and Fraction II (C).

3.3. Off-line nanoflow HPLC/MALDI MS

The nano-LC/UV chromatogram of the nucleopeptide sample digested with nuclease P1 (Fig. 3A) indicates the presence of two relatively low abundant species with retention times of roughly 13 and 17 min. The baseline absorbance in this chromatogram gradually decreases with increasing time as acetonitrile from solvent B (50% acetonitrile with 0.1% TFA) replaces water from solvent A (0.1% TFA in water); the sensitivity is low (compared with a conventional UV-detector) because of the nanoflow HPLC system's short path length (30 μm). The

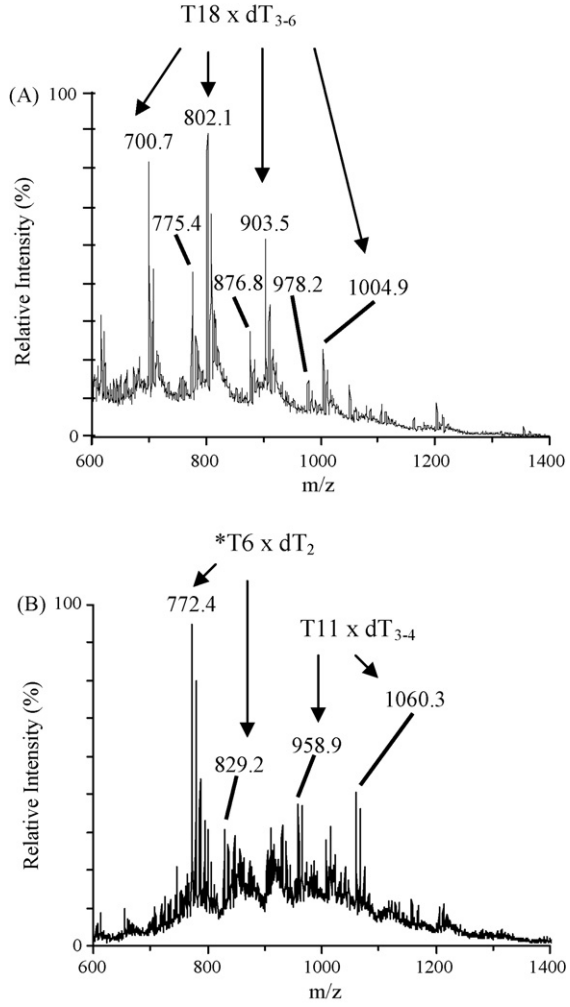

Fig. 2. Nano-ESI-MS analysis of HPLC-purified nucleopeptides. Nanospray spectra of HPLC-purified (Fig. 1A) nucleopeptides Fractions I (A) and II (B).

MALDI spectrum of Fraction 1 (Fig. 3B) shows peaks corresponding to T18 × dT_2 and T18 × dT_3 (*m/z* 1796.1 and 2100.3, respectively), and the MALDI spectrum of Fraction 2 (Fig. 3C) contains an intense peak corresponding to $T6_{(-5)}$ × dT_2 (*m/z* 2485.5).

3.4. On-line microflow HPLC/ESI-MS

The total ion chromatogram of the nucleopeptide mixture produced by on-line microflow HPLC/ESI (Fig. 4A) exhibits peaks corresponding to a large number of compounds. Despite the limited degree of separation evident in the chromatogram, nucleopeptides were found in the eluate coming off the column at 21.5, 23.6, and 24.9 min. The mass spectra recorded at these retention times (Fig. 4B–D) contain prominent peaks corresponding to doubly and triply protonated T18 × dT_2 (*m/z* 898.5 and 599.3, respectively), T11 × dT_2 (*m/z* 1285.7 and 857.5, respectively), and $T6_{(-3)}$ × dT_2 (*m/z* 1352.4 and 901.9, respectively).

3.5. On-line nanoflow HPLC/ESI-MS

The reconstructed nanoflow LC/ESI-MS chromatogram (Fig. 5) and the corresponding mass spectra (not shown) indicate that the nucleopeptides $T6_{(-3)}$ × dT_2, $T6_{(-5)}$ × dT_2, $T6_{(-7)}$ × dT_2, T11 × dT_2, and T18 × dT_2 were clearly separated from a nuclease P1 digested nucleopeptide sample. Each of the five nucleopeptide peaks observed in the chromatogram was generated by plotting only the doubly charged molecular ion signals for the species mentioned above.

4. Discussion

The ultimate outcome of a mass spectrometric analysis of a peptide–nucleic acid complex is identification of those amino acids that are crosslinked to nucleic acids. Such data provides insight into the mechanism by which the protein interacts with its nucleic acid substrate. The work presented here was part of a biochemical project that was conducted to map the DNA-binding domain of *E. coli* uracil-DNA glycosylase.

Uracil-DNA glycosylase is a monomeric, 228 amino acid polypeptide (Mr = 25,562 Da) that initiates the uracil-excision DNA repair pathway *in vivo* by cleaving the N-glycosylic bond between uracil and deoxyribose in DNA [23]. Ung shows a two-fold substrate specificity for uracil residues in single-stranded as opposed to double-stranded DNA [23]. While all amino acids have comparable UV crosslinking potentials, nucleic acids have distinctly different crosslinking potentials; specifically, thymidine and uridine, respectively, are by far the most efficient deoxynucleotide and ribonucleotide crosslinkers [3]. The UV-crosslinking specificity of polydeoxythymidine towards amino acids is so low that virtually any of a DNA-binding protein's amino acids in contact with a thymine base can take part in crosslinking. This lack of specificity increases the number of crosslinks that can form within the protein–nucleic acid interface.

Previously when oligodeoxythymidine dT_{20} was used as a DNA substrate, three tryptic peptides (T6, T11 and T18) were shown to be involved in UV crosslinking [6,10,19]. This finding relied on the isolation of crosslinked peptide (nucleopeptides) by anion exchange chromatography following the in-gel trypsin digestion of the nucleoprotein complex. Altogether, these previous studies revealed five dT_{20} nucleopeptides by MALDI mass spectrometry in a fraction eluted with 1 M TE buffer from the anion exchange column (spectrum not shown). Two of the nucleopeptides were identified as T11 × dT_{20} and T18 × dT_{20}, while the other three contained the T6 peptide minus 3, 5 and 7 amino acids from the Ung C-terminal. Nuclease P1 digestion, employed to remove most of the oligonucleotide portion of these complexes and thereby enhance the nucleopeptide signal in a positive ESI or MALDI mass spectrum, compounded sample complexity. In the present study, complex mixtures of dT_n (n = 2–6) nucleopeptides were isolated, and various separation configurations coupled either off-line or on-line with ESI and MALDI mass spectrometry were surveyed. The goal of this survey was to find which configuration allowed for a complete mass spectrometric

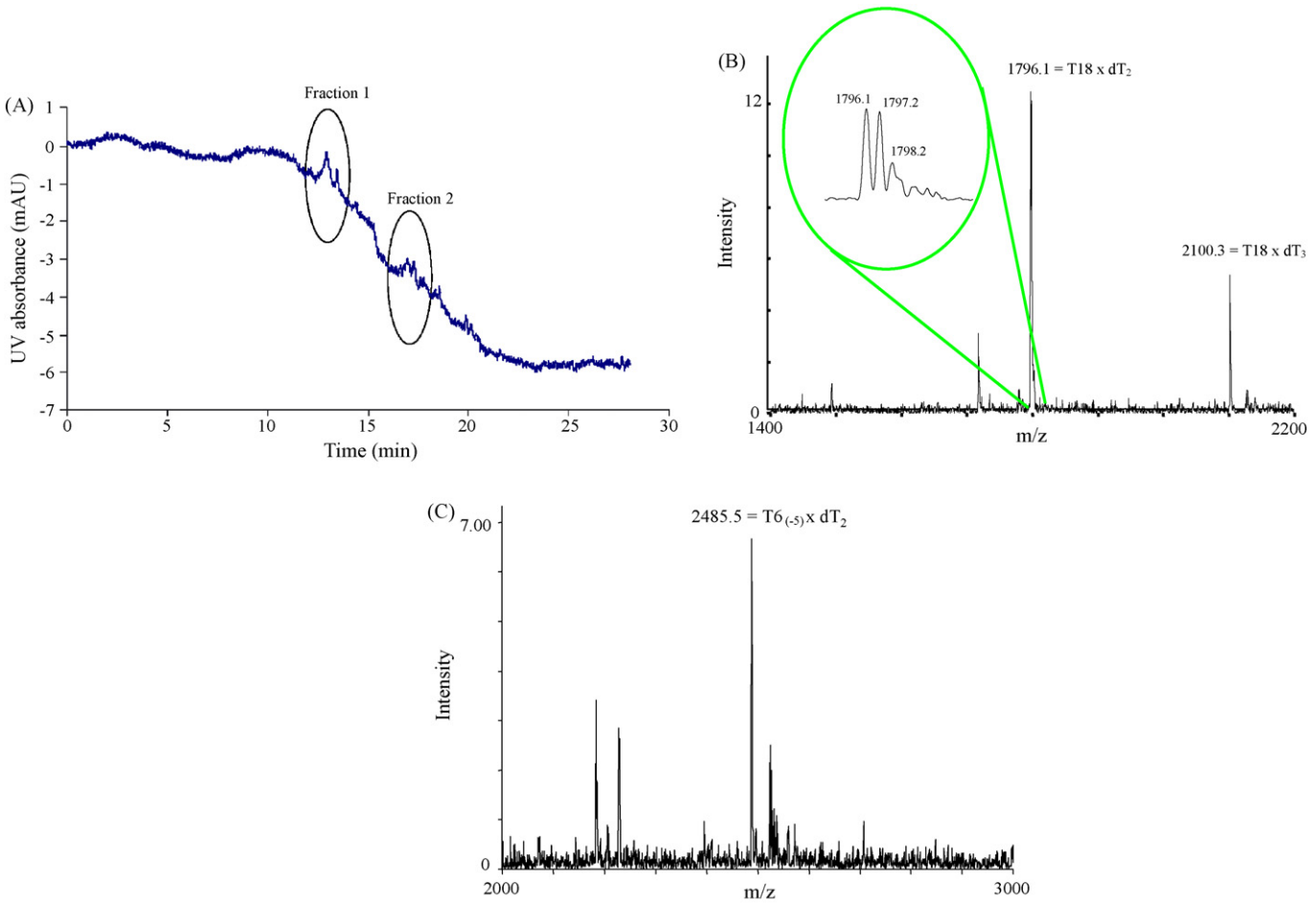

Fig. 3. Off-line nano-LC isolation of nucleopeptides. Nano-LC/UV chromatogram produced by loading the column with 10 μL of a nuclease P1 digest (A). The sample was eluted at 0.5 μL/min (pressure at inline filter = 100 bar) with an exponentially produced gradient of 0% to ~100% solvent B (0.1% TFA in 40% acetonitrile). UV detection was at 200 nm. Two fractions were collected manually with a fraction volume of 2 μL (2 min collection period). One microliter of solvent A (0.1% TFA in water) was added to each vial before starting the manual collection. An aliquot (1 μL) of each of the purified nucleopeptide samples was mixed with THAP matrix in a 1:3 ratio and analyzed by MALDI MS. MALDI spectrum exhibiting signals corresponding to T18 × dT_2 (*m/z* 1796.1) and T18 × dT_3 (*m/z* 2100.3) nucleopeptides isolated in nano-LC/UV Fraction 1 (B); the inset shows the isotopic distribution of T18 × dT_2. MALDI spectrum of $T6_{(-5)}$ × dT_2 (*m/z* 2485.5) nucleopeptide isolated in nano-LC/UV Fraction 2 (C).

characterization of a nucleopeptide mixture. The small sample quantities isolated from the crosslinking experiments required the investigation of high sensitivity ESI configurations, namely nanospray/, micro-LC/, and nano-LC/ESI-MS. These off-line and on-line microflow/nanoflow LC/MS interfaces were evaluated for their ability to provide sample separation and mass spectrometric detection of the five dT_2 nucleopeptides known to be present.

Initial fractionation of the dT_n nucleopeptide mixture was performed by off-line microflow chromatography and two fractions (labeled I and II in the LC/UV chromatogram shown in Fig. 1A) were subsequently analyzed by MALDI and nanoelectrospray MS. The MALDI spectrum of the first fraction revealed the presence of five T18 nucleopeptides with the general formula T18 × dT_n (n = 2–6), as well as four T18 nucleopeptides (n = 3–6) without a phosphate group. Fraction II, isolated during the same preparative run, contained seven nucleopeptides, four of which derived from the T6 peptide and three from T11. Clearly, the five dT_2 nucleopeptides in question could not be isolated by off-line microflow LC fractionation.

The reported advantages of nanoelectrospray tandem mass spectrometry, in particular the technique's subpicomole sensitivity for peptides and facile acceptance of submicroliter volumes of sample [20,22], made it an attractive candidate for analyzing isolated nucleopeptides. However, we found that HPLC enriched fractions of nucleopeptides produced very complicated nanospray spectra (Fig. 2A and B). The complexity of these spectra arises partly from the multiple charge states of the individual nucleopeptides that are characteristic of electrospray ionization. In general, the charge states of a nucleopeptide were between +2 and +4, usually with the +3 charge state being most abundant. Additional complexity in these spectra came from adduction primarily of sodium and potassium. Leaching from the nanospray needles by the acidic HPLC fractions could explain the abundant presence of the latter in the nanospray spectra. The combination of multiply charged states and cation adduction greatly

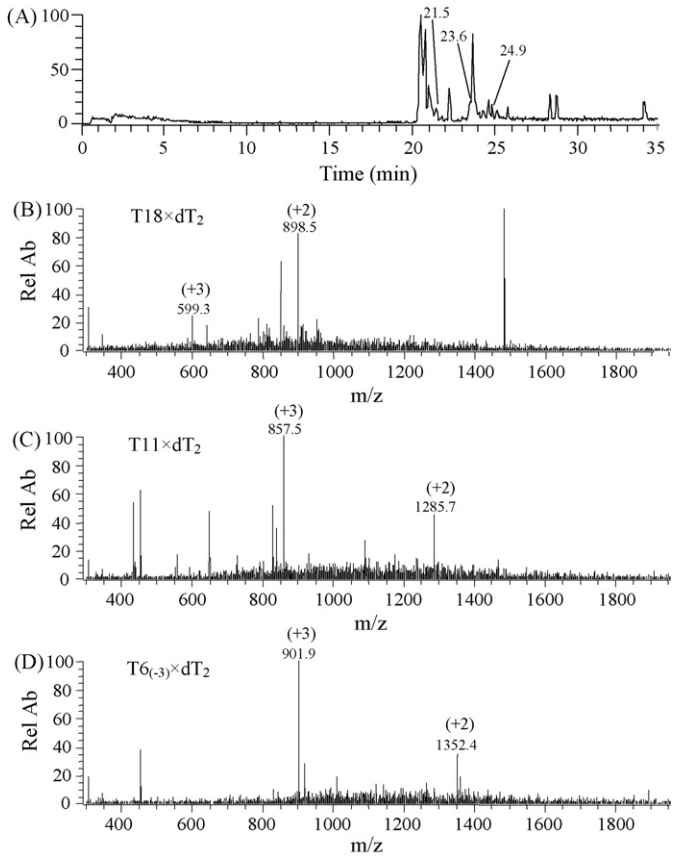

Fig. 4. Online micro-LC/ESI-MS of a nucleopeptide mixture. Base-peak chromatogram of a nucleopeptide mixture was produced using a micro-HPLC system coupled on-line with an ESI ion trap mass spectrometer (A). Individual mass spectra recorded at retention times of 21.5 (B), 23.6 (C), and 24.9 min (D), respectively.

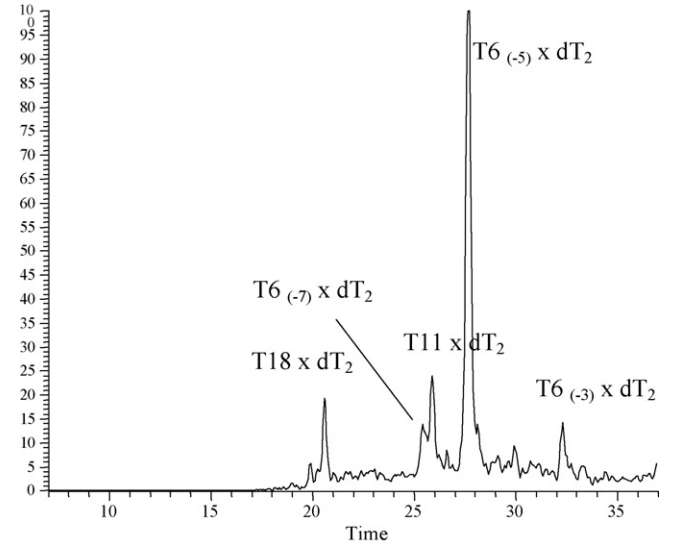

Fig. 5. Nano-LC/ESI-MS mass-chromatogram of P1 digested nucleopeptides. The chromatogram was reconstructed from *m/z*-signals corresponding to doubly charged molecular ions. The nucleopeptide sample (5 μL) was injected onto the column (75 μm (i.d.) × 40 cm long; 5 μm C_{18}) and eluted at 0.3 μL/min (pressure at inlet filter = 140 bar) with an exponentially produced gradient of 0% to ~100% solvent B (0.1% AA plus 0.01% TFA in 40% acetonitrile). Ion detection was from *m/z* 400 to 2000. All five dT_2 nucleopeptides were detected using this method.

Table 1
Ung × dT20 crosslinked nucleopeptides detected by various LC/MS interfaces

LC/MS method	Nucleopeptides				
	T6-3	T6-5	T6-7	T11	T18
Off-line microflow MALDI-TOF	√	√	√	√	√
Off-line microflow nano-ESI-MS	–	√	√	√	√
Off-line nanoflow MALDI-TOF	–	√	–	–	√
On line microflow ESI-MS	√	–	–	√	√
On line nanoflow ESI-MS	√	√	√	√	√

reduced the technique's sensitivity for single nucleopeptide ion species.

The utility of peptide fractionation by nanoscale LC before MALDI or nano-ESI has been reported [22]. Even though the sensitivity of the UV detection used for guiding the collection of fractions containing nucleopeptides was limited in the present study by a short path-length (30 μm), this mode of sample fractionation allowed relatively pure dT_2 nucleopeptide species, viz. T18 × dT_2 and $T6_{(-5)}$ × dT_2 (Fig. 3B and C) to be isolated. However, the T11 × dT_2 nucleopeptide was not detected using this approach.

The on-line LC/ESI-MS approaches employed in this study fully revealed the complexity of the isolated dT_n nucleopeptide mixtures. A typical micro-LC/ESI base-peak chromatogram is presented in Fig. 4A. Even though the majority of the peaks present in this chromatogram do not correspond to predicted dT_2 nucleopeptides, three of them do (Fig. 3B through C). The charge state distribution of the nucleopeptide species observed during micro-LC/ESI/MS experiments are similar to those observed for nanospray ESI experiments (charge states distributed from +2 to +4, with the +3 charge state being most abundant), but cation adduction is not observed. All three of the dT_2 nucleopeptides (T6, T11 and T18) that define the DNA-binding domain of Ung were completely separated using this online approach.

Nanoscale LC/MS analysis improved sensitivity and chromatographic resolution sufficiently to provide a comprehensive nucleopeptide analysis of all dT_2 species. ESI-MS spectra of the five dT_2 nucleopeptides known to exist in the sample were acquired during a 40 min chromatographic run performed on a 40 cm long nanoscale column (Fig. 5) with each nucleopeptide producing a distinct chromatographic peak 15–20 s wide. Furthermore, this method facilitated the acquisition of collision-induced dissociation spectra (CID) for all of the dT_2 nucleopeptides, thus allowing the individual amino acids responsible for the interaction between Ung and the DNA substrate to be located in a single chromatographic run.

Table 1, which summarizes the results obtained from each LC/MS method, indicates which nucleopeptides were detected by each approach.

5. Conclusions

In general, on-line LC mass spectrometric analysis of nucleopeptides is considerably less tedious and time consuming than off-line approches and minimizes cation adduction associated with nano-ESI-MS. In this study, the nanoscale LC format of

LC/MS-ESI proved to be the best method for characterizing nucleopeptides, by virtue of being able to analyze an entire mixture in a single analysis.

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