Enrichment of Protein–RNA Crosslinks from Crude UV-Irradiated Mixtures for MS Analysis by On-Line Chromatography Using Titanium Dioxide Columns

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ABSTRACT:

UV crosslinking is an appropriate method to identify proteins that directly contact nucleic acid, e.g., RNA. In combination with modern mass spectrometric (MS) analysis such an approach provides the opportunity to reveal not only the nature of the crosslinked proteins but also to identify the actual crosslinking sites between the protein and the nucleic acid. However, the relatively low yield in UV-induced crosslinking makes it difficult to identify in particular those species by MS that represent peptide–nucleic acid conjugates, as the great excess of noncrosslinked material interferes with their detection in MS. Here, we present an automated enrichment strategy of crosslinked peptide–RNA oligonucleotides derived from crude mixtures of UV-irradiated ribonucleoprotein (RNP) particles that uses TiO_2 columns integrated within a two-dimensional (2D) nanoliquid chromatography (LC) system. The setup combines two C18 precolumns, a TiO_2 enrichment column and a nanoanalytical column. It allows the removal of the noncrosslinked RNA and protein moiety and the specific enrichment of crosslinked peptide–RNA conjugates so that UV-irradiated and subsequently completely hydrolyzed RNP complexes can directly be loaded and analyzed by MS. In this feasibility study, we demonstrate the specific enrichment of peptide–RNA oligonucleotides derived from UV-irradiated native spliceosomal U1 snRNPs and spliceosomal [15.5K-61K-U4atac snRNA] complex reconstituted in vitro. © *2009 Wiley Periodicals, Inc. Biopolymers 91: 297–309, 2009.*

Keywords: 2D liquid chromatography; crosslinking; protein; RNA; titanium dioxide

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INTRODUCTION

Ribonucleoprotein (RNP) particles play essential roles in a number of fundamental cellular processes, including pre-mRNA splicing.[1–4] Detailed knowledge of contact sites between proteins and RNA within the RNP is crucial for a profound understanding of their functions. In the absence of high-resolution structures of protein–RNA complexes, UV crosslinking at 254 nm combined with mass spectrometry (MS) is a powerful and straightforward tool to identify such contact sites in purified native RNPs.[5,6] However, a bottleneck of this approach has always been the low yield of protein–RNA crosslinking, when nonmodified RNA—as present in native particles—is used. Therefore, the purification of the crosslinked species over the excess of noncrosslinked peptide and RNA moieties is the essential step in the analysis.

In recent years we have used size-exclusion (SE) chromatography of UV-irradiated and subsequently hydrolyzed

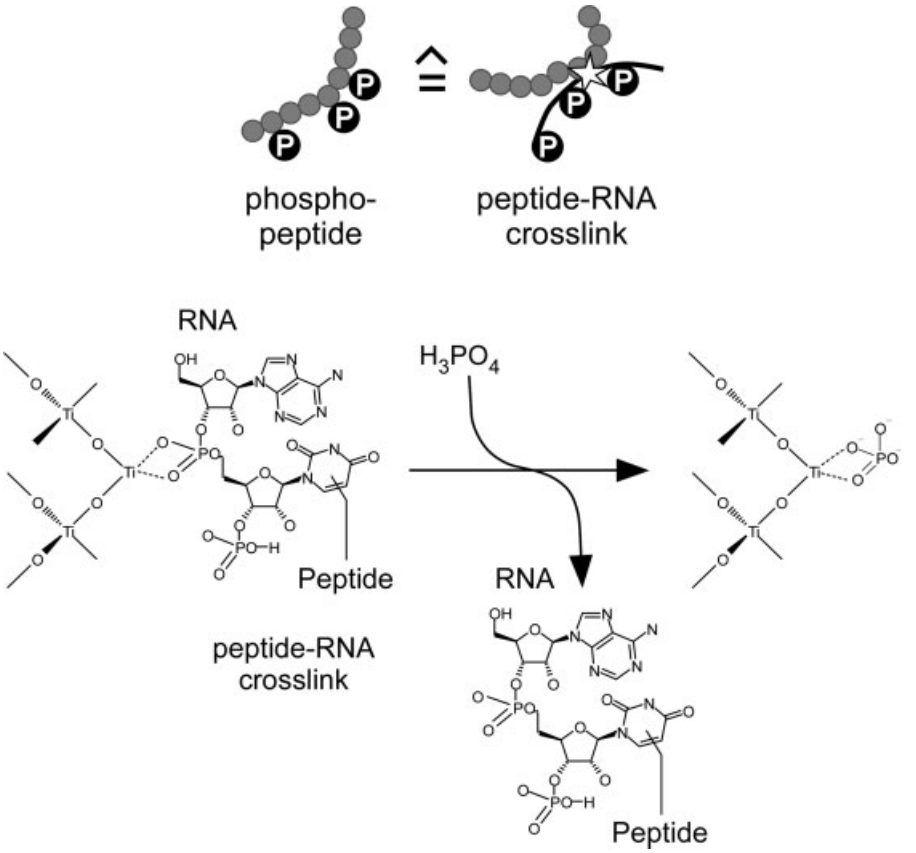

FIGURE 1 Purification strategy of peptide–RNA oligonucleotide crosslinks using TiO_2 and inorganic phosphate for replacement of bound crosslinks. The purification took advantage of the fact that peptide–RNA crosslinks resemble phosphopeptides.

RNPs with endoproteinases as a first step to separate the peptides that are crosslinked to RNA from the great excess of noncrosslinked peptides.[7–9] After hydrolysis of the RNA, peptide-linked oligonucleotides could be purified by microbore, capillary, or nano-reversed-phase (RP) high-pressure liquid chromatography (HPLC). For MS-based sequence determination, the conjugates were detected by (i) their absorbance at 220 and 254 nm,[6] (ii) precursor-ion scanning combined with multiple reaction monitoring,[10] or (iii) additional enrichment by immobilized metal affinity chromatography.[5,11,12]

Here, we report an automated enrichment strategy of peptide–RNA oligonucleotides derived from UV-irradiated and hydrolyzed snRNPs based on titanium dioxide (TiO_2)[13] columns integrated within a 2D nano-LC system. The system avoids the separation of the noncrosslinked peptide moiety in the previous off-line SE step. TiO_2 has been reported to be highly suitable for the enrichment of phosphopeptides as the copurification of acidic peptides is minimized in comparison with enrichment strategies using IMAC.[14–16] Since phosphopeptides and peptide–RNA oligonucleotides share similar properties (i.e., the phosphate group) TiO_2 should also prove useful in the enrichment of the latter species (Figure 1). Furthermore, TiO_2 can be integrated into a HPLC system, whereas IMAC agarose beads do not withstand HPLC conditions. Accordingly, several systems have been set up for the on-line enrichment of phosphopeptides with TiO_2 columns.[17–21]

Our on-line enrichment strategy of peptide–RNA oligonucleotides from complex mixtures differs from the TiO_2 on-line set-ups reported earlier, as it includes an additional desalting step. It combines two C18 trapping columns, a TiO_2 enrichment column and a C18 nanoanalytical column. Enriched peptide–RNA oligonucleotides are spotted onto MALDI targets and analyzed by MALDI-ToF/ToF according to Kühn-Hölsken et al.[6] In this feasibility study, we tested the system for the enrichment, detection, and sequencing of peptide–RNA crosslinks derived from UV-irradiated native U1 snRNPs and from the in vitro reconstituted [15.5K-61K-U4atac snRNP] complex; detection was performed without any further off-line chromatographic separation steps. Owing to the reduction in the number of experimental steps the loss of material is minimized. Moreover, this procedure

makes possible the enrichment and subsequent analysis of phosphopeptides derived from noncrosslinked RNPs without a change in the set-up.

RESULTS

Loading, Washing, and Elution Conditions Suitable for TiO_2 On-Line Chromatography Conditions for the Enrichment of Peptide–RNA Crosslinks and Phosphopeptides

We first established loading and elution conditions of crosslinks on TiO_2 beads with the aim of finding conditions that can be used in a multidimensional chromatography set-up coupled to MALDI-ToF MS. Recent studies demonstrated that distinct loading, washing, and elution conditions are crucial for the successful enrichment of phosphopeptides using TiO_2.[13,22] Loading in the presence of 2,5 dihydroxybenzoic acid (DHB) at a very low pH [5% trifluoroacetic acid (TFA)] has been shown to be optimal for the specific enrichment of phosphopeptides[22] in an off-line approach. Washing was performed once with loading followed by a washing buffer, excluding DHB, and then phosphopeptides were eluted with NH_4OH (25%, pH 10.5). In alternative on-line approaches, phosphopeptides were readily eluted upon autosampler injection of NH_4OH at pH 10.5.[20,21]

However, these off-line and on-line conditions were—in our hands—not compatible with any nano-LC system that used standard RP materials and fused silica capillaries as flow connectors.

We thus tested suitable conditions for TiO_2 enrichment that were more suitable for our purpose. We first used an off-line (batch) system with an appropriate volume of TiO_2 slurry. For these initial feasibility studies, we monitored the enrichment of phosphopeptides derived from the U1 snRNP specific protein 70K after in-gel digestion[23] under various elution conditions [10 mg/ml methylphosphonic acid; 10 mg/ml glucoseamine-6-phosphate; 100 m*M* ATP; 10 mg/ml $(NH_4)_2HPO_4$]. In addition, we tested for the enrichment of a previously studied peptide–RNA crosslink derived from the U1 70K protein after UV irradiation of U1 snRNPs. For this purpose, U1 70K peptide–RNA oligonucleotide conjugates were isolated in a semipreparative manner by microbore HPLC fractionation as described by Kühn-Hölsken et al.[6] Since these fractions still contain a large number of residual contaminating noncrosslinked peptides in addition to the actual crosslinked species, this serves as an ideal sample to test for enrichment and recovery of the crosslink. We also tested for the enrichment of that particular crosslink from a UV-irradiated U1 snRNP complex that was digested in solution with RNases and endoproteinase and desalted with stage tips before TiO_2 enrichment.

We found that best results were obtained by loading the sample in 50% (v/v) acetonitrile (ACN) and 0.25% (v/v) TFA in water, stepwise washing with 25 and 50% (v/v) ACN/0.25% (v/v) TFA in water and final elution of the phosphopeptides and crosslinks in 10 mg/ml $(NH_4)_2HPO_4$ pH 1.1 in water combined with a subsequent desalting step using stage tips.[24] We observed satisfactory enrichment and detection of a U1 70K phosphopeptide (YDERPGPpSPLPHR, data not shown) and the U1 70K crosslink (RVLVDVER to an AU dinucleotide to an AUCAC pentanucleotide, Figures 2A and 2B).

It has been reported that TiO_2 has certain disadvantages in the enrichment of multiply phosphorylated or very hydrophilic species (e.g., short tryptic fragments), as the former bind strongly, and the latter fail to bind at all, to the TiO_2 matrix.[16] However, we do not observe a similar effect upon the enrichment of the U1 70K peptide–RNA crosslink. Although the 70K peptide that is crosslinked to a dinucleotide (AU) is eluted more efficiently when its signal intensity is compared to those of the other crosslinks before and after enrichment, crosslinks carrying three, four, and five nucleotides are eluted equally well from the TiO_2 material (Figures 2A and 2B). Therefore, the intensity of the shorter oligonucleotide modified peptides is relatively increased as compared to the nonenriched HPLC fraction. In the same manner, it was possible to enrich the 70K specific peptide RVLVDVER crosslinked to an di- and trinucleotide from the crude mixture of in-solution digested UV-irradiated U1 snRNPs, demonstrating the capacity in the removal of the background (Figures 2C and 2D). We conclude that for the successful enrichment of the crosslinked species from a crude mixture, i.e., a completely hydrolyzed RNP, the (crosslinked) RNA moiety should be as small as possible which can be achieved by digestion of the RNA with a mixture of RNase A, T1, and benzonase.

2D Chromatographic Setup

The conditions for loading, washing, and elution to enrich peptide–RNA crosslinks are integrated into our multidimensional chromatographic set-up by using TiO_2 columns packed in our laboratory. Figure 3 shows the assembly of the different columns mounted into the chromatography system equipped with two 10-port nanovalves, and it illustrates the individual chromatographic steps. U1 snRNP and reconstituted [15.5K-61K-U4 snRNA] complex[25,26] were hydrolyzed with ribonucleases (RNase A, T1, and benzonase) and endoproteinases (trypsin or chymotrypsin).

The crude mixture of noncrosslinked and crosslinked peptides, phosphorylated peptides, and noncrosslinked RNA oli-

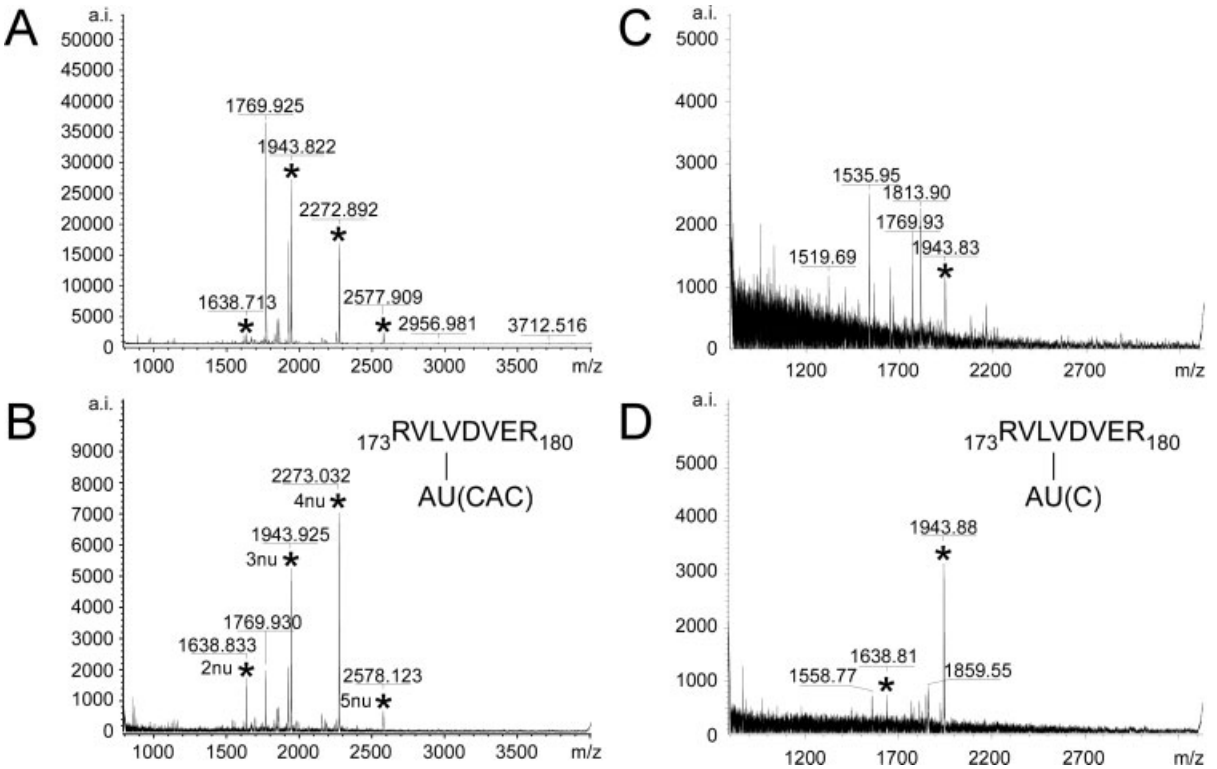

FIGURE 2 Test for appropriate loading, washing, and elution conditions for enrichment of peptide–RNA crosslinks on TiO_2 beads using liquid chromatography (LC)-compatible solvents. A: MALDI peptide mass fingerprint of a capillary RP-HPLC fraction containing semipreparatively purified U1 70K peptide RVLVDVER (positions 173 to 180) crosslinked to AU (m/z = 1638.713), AUC (m/z = 1943.822), AUCA (m/z = 2272.892), and to AUCAC (m/z = 2577.909). Crosslinks are marked with an asterisk. The sequence of the crosslinked peptide and RNA was determined by MSMS analysis (see Figure 6). B: MALDI peptide mass fingerprint of the same fraction after enrichment of the crosslinks with TiO_2. The number of nucleotides of the crosslinked RNA moiety is listed within the spectrum. C: MALDI peptide mass fingerprint of UV-irradiated U1 snRNPs that were completely hydrolyzed with RNases and endoproteinase trypsin and desalted before analysis. D: MALDI peptide mass fingerprint of UV-irradiated U1 snRNPs that were completely hydrolyzed with RNases and endoproteinase trypsin and putative crosslinks being enriched with TiO_2 subsequent to the desalting of the sample before analysis.

gonucleotides is injected onto the first trapping column (C18 Vydac, 10 mm × 0.15 mm) in the presence of 3.5% (v/v) ACN, 0.1% TFA in water at a flow rate of 5 μl/min for 25 min. Under these conditions the vast majority of the RNA oligonucleotides does not bind to the trapping column and is discarded into the waste. Noncrosslinked and crosslinked peptides bind to the C18 trapping column mounted between the two 10-port valves (Figures 3A and 3B). After the two valves were switched from position 1-10 and 1-2 to 1-2 and 1-10, respectively, the nanopump elutes the bound species from the C18 trapping column by applying a gradient from 8 to 48% (v/v) ACN (10 to 60% solvent B_1), 0.25% (v/v) TFA in water in 120 min with a flow rate of 300 nl/min. Under these conditions, species that carry a phosphate moiety (i.e., phosphorylated peptides and peptide-linked RNA oligonucleotides) are bound effectively to the TiO_2 column (equilibrated with 48% ACN, 0.25% TFA in water at a flow rate of 3.5 μl/min), whereas most of the noncrosslinked and nonmodified species do not bind and are subsequently eluted and separated on the C18 analytical nanocolumn (Vydac 5 μm particle size, 300 Å pore width, 120 mm × 0.075 mm, Figures 3C and 3D) and analyzed by MALDI-MSMS.

Peptides eluted from the analytical column were mixed with 2,5-DHB (10 mg/ml in 20% (v/v) ACN, 0.1% (v/v) H_3PO_4) and were spotted every 30 s onto a MALDI stainless steel target and peptides in the spot fractions were analyzed by automated MS and MSMS on a 4800 MALDI-ToF/ToF instrument (Sciex Applied Biosystems) or spotted onto a

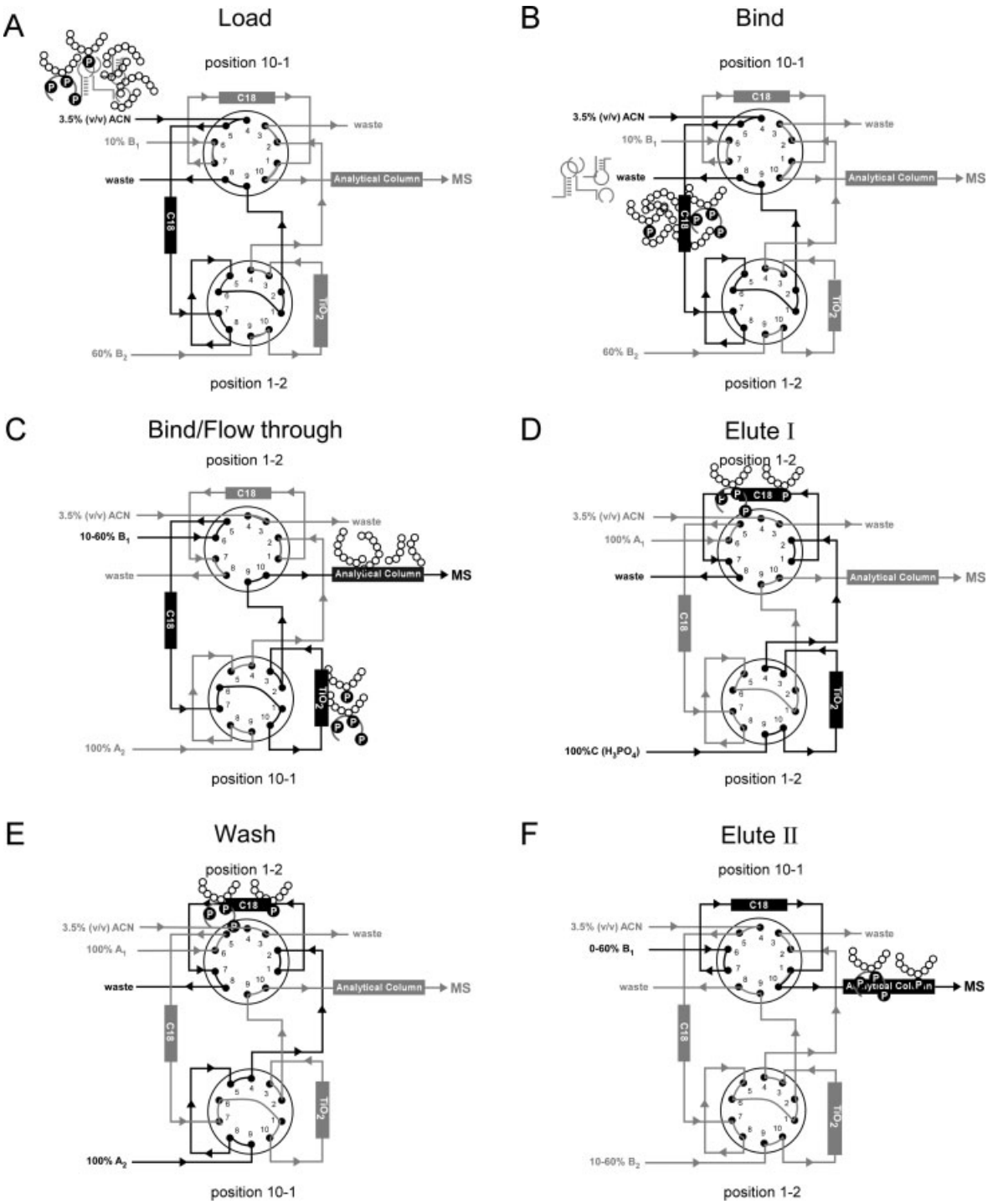

FIGURE 3 Schematic overview of 2D chromatography setup in the different 10-port valve positions during automated simultaneous enrichment of phosphopeptides and peptide–RNA crosslinked derived from (UV-irradiated) protein–RNA complexes after complete hydrolysis of the protein and RNA moieties. Active connections and pumps with the applied solvents in the respective steps of the chromatography are shown in black, those that not used are in gray. A: Loading of the crude mixture onto the first C18 precolumn. B: Binding of phosphopeptides, crosslinked peptide-RNA oligonucleotides, and the excess of noncrosslinked peptides to the first C18 precolumn and elution of the excess of noncrosslinked RNA oligonucleotides from the system (into the waste). C: Elution of the trapped mixture from the first C18 precolumn, and binding of phosphopeptides and peptide–RNA crosslinks to the TiO_2 column. Nonphosphorylated and noncrosslinked peptides did not bind and were separated by the nanoanalytical column and subsequently analyzed by MS. D: Elution of bound phosphopeptides and crosslinks from the TiO_2 column onto the second C18 precolumn for desalting. E: Washing of the phosphopeptides and crosslinks on the C18 precolumn. F: Elution of the phosphopeptides from the C18 desalting column onto the nanoanalytical column for subsequent separation and analysis in the mass spectrometer.

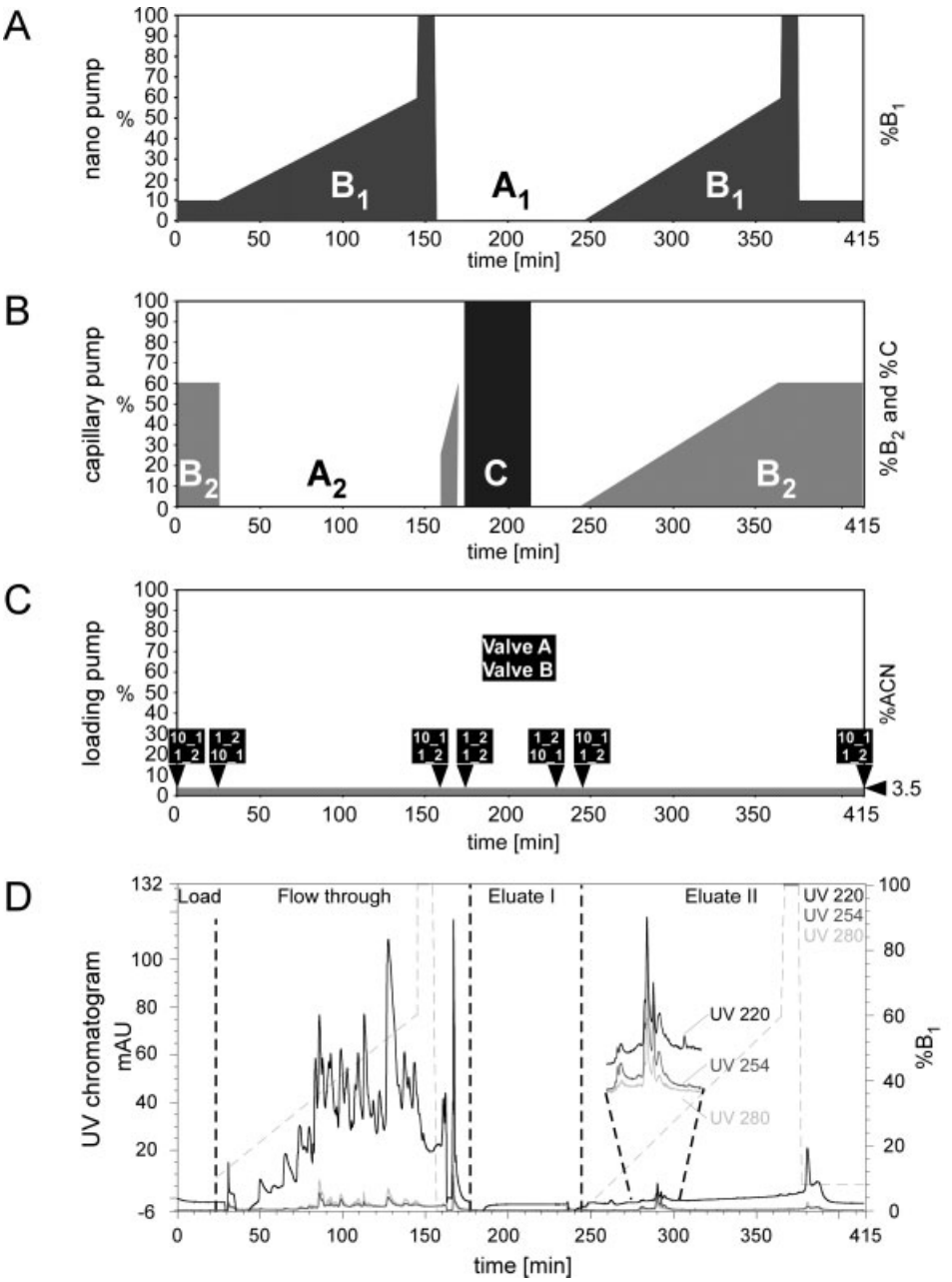

FIGURE 4 Applied gradients of the nano, capillary, and loading pumps during 2D chromatography including time points of switching of the 10-port valves. The elution profiles were monitored by the connected UV detector. The overall time of separation was 415 min. A: Gradients generated by nanopump at 300 nl/min flow rate for elution of the nonphosphorylated and noncrosslinked species from 25 to 145 min (flow-through) and for elution of the phosphorylated and crosslinked species between 245 and 365 min (elution II). Solvent A_1 was 0.25% (v/v) TFA and solvent B_1 was 80% (v/v) ACN, 0.25% (v/v) TFA. B: Applied solvents and gradients of the capillary pump for equilibration of the TiO_2 column and washing and elution of the bound species on and from the TiO_2 column (Eluate I), respectively using solvents A_2 and B_2 (see A_1 and B_1) and C. The flow rate was 3.5 μl/min. A_2 was used to equilibrate the second C18 precolumn and subsequently desalt the bound phosphopeptides on the second C18 precolumn. C: Isocratic profile of the loading column. The switches of the two 10-port valves at various time points are indicated. D: UV chromatogram of the eluate in the different 2D separation steps. The dashed line indicates the applied gradients for separation of noncrosslinked peptide moiety (flow-through) and the crosslinked species (Eluate II).

Bruker anchor chip and analyzed on a Bruker Reflex IV. Alternatively, LC-separated peptides were mixed with α-cyano-4-hydroxy-cinnamic acid (α-cyano, 10 mg/ml in 70% (v/v) ACN, 0.1% (v/v) TFA) and collected onto stainless steel MALDI targets suitable for automated MS and MSMS analysis on a 4800 MALDI-ToF/ToF instrument. After switching of Valve 2 to Position 1-2, the TiO_2 column is washed according to the procedures in our off-line batch experiments with a gradient of 20–48% (v/v) ACN, 0.25% (v/v) TFA in water that is delivered by the capillary pump (channels A_2 and B_2 at a flow rate of 3.5 μl/min). Peptides bound to the TiO_2 column were eluted by the third channel of the capillary pump with solvent C (10 mg/ml $(NH_4)_2HPO_4$ pH 1.1 in water) at a flow rate of 3.5 μl/min onto the second trapping column mounted at Valve 1 (Figure 3D). This trapping column contains ReproSil-Pur Basic C18-HD (5 μm, Dr. Maisch GmbH) combining a small particle diameter with similar binding properties as Oligo R3 material (Applied Biosystems) which has been shown to be most suitable for the desalting of enriched phosphopeptides before MS analysis.[27] After switching Valve 2 back into position 10-1 bound phosphopeptides and crosslinks are washed with solvent A_2 [0.25% (v/v) TFA in water] delivered at a flow rate of 3.5 μl/min by the capillary pump (Channels 1 and 2) for 30 min (Figure 3E). After switching Valve 2 back to Position 1–2 and Valve 1 to Position 10–1, phosphopeptides and crosslinks are eluted from the trapping column and separated on the analytical column with a gradient from 0 to 48% (v/v) ACN, 0.25% (v/v) TFA in water in 120 min at a flow rate 300 nl/min. The TiO_2 column is re-equilibrated by the capillary pump for the next sample by applying a gradient of 0 to 48% (v/v) ACN, 0.25% (v/v) TFA in water in 120 min at a flow rate of 3.5 μl/min. The phosphopeptides and crosslinked species eluted are mixed with 2,5-DHB as matrix and are spotted every 30 s onto a MALDI target. Spots were analyzed by MALDI MS and MSMS. In those fragment spectra that show a loss of H_3PO_4 (98 a.m.u.) the corresponding precursors are considered to be phosphopeptides or peptide–RNA crosslinks and are reanalyzed manually by MSMS.

Figure 4 summarizes the solvent gradients applied to the nanopump for separation of the nonmodified peptides and modified peptides (Figure 4A), to the capillary pump for washing and elution of the crosslinks on and from the TiO_2 column (Figure 4B) and to the loading pump for loading the sample including applied switches of the valves (Figure 4C). Figure 4D shows the elution profile of the first gradient for the separation of the noncrosslinked species (25–145 min) and the second gradient for the separation of the crosslinked

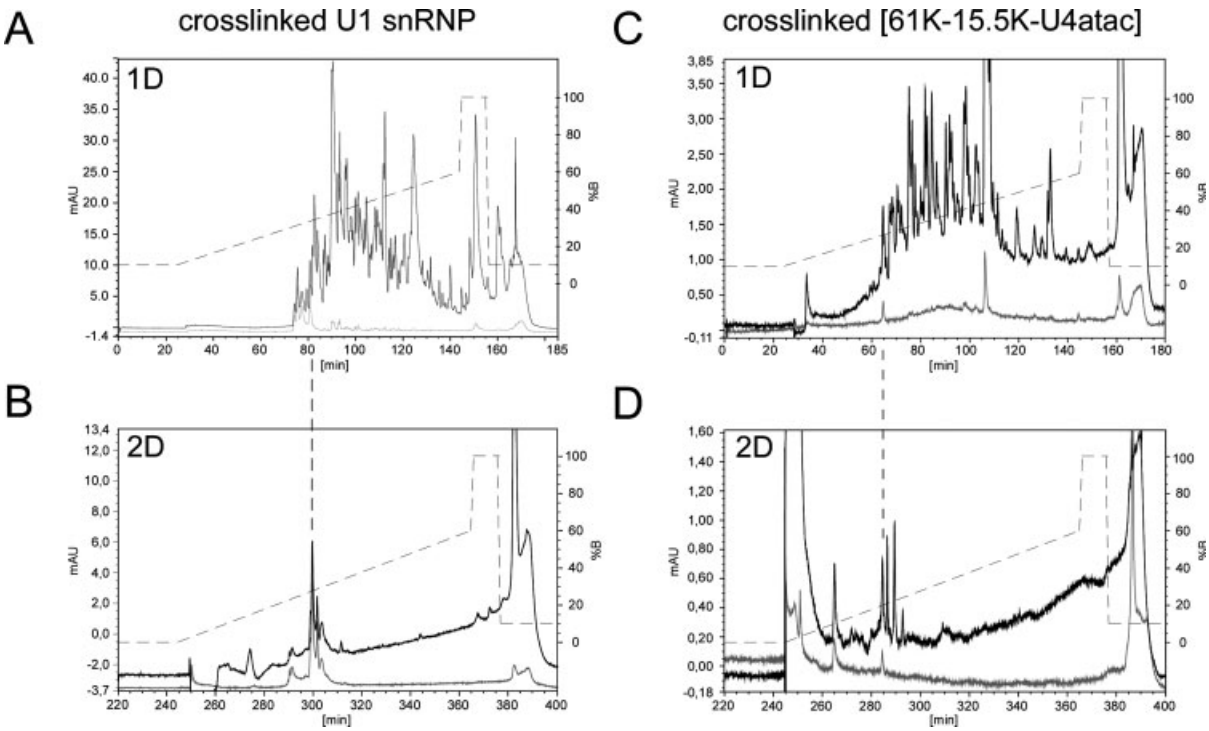

FIGURE 5 Comparison of standard 1D (A and C) and 2D chromatography (B and D) in the separation of crosslinked peptide–RNA oligonucleotide conjugates derived from UV-irradiated U1 snRNP and [15.5K-61K-U4atac snRNA] hydrolyzed with RNases and endoproteinases.

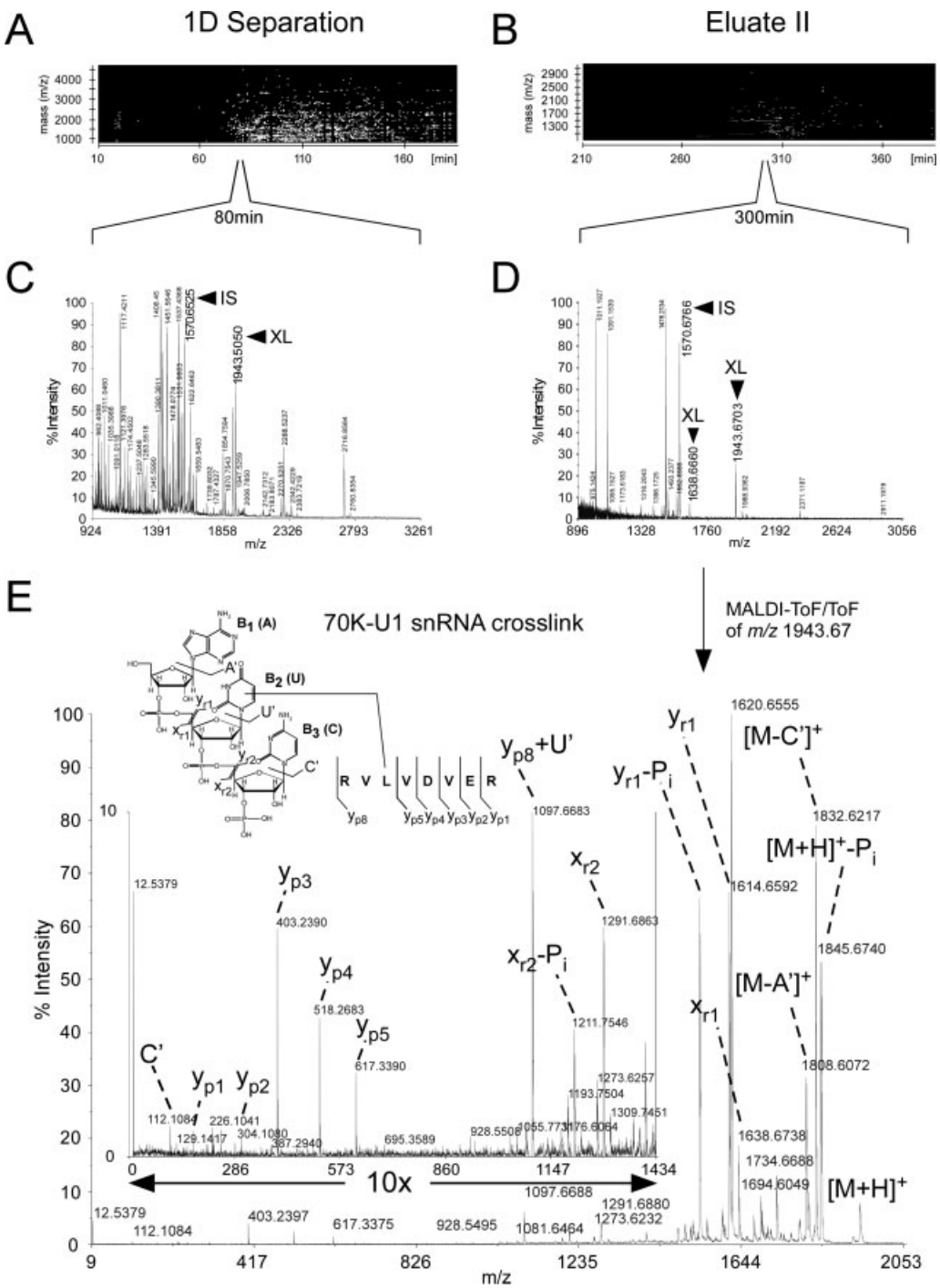

FIGURE 6 MALDI MS and MSMS analysis of enriched crosslinked peptide–RNA oligonuclotides derived from UV-irradiated U1 snRNPs hydrolyzed with RNases and endoproteinase. A and B: "Heat maps" of the 1D separation and the Eluate II fraction of the 2D separated mixture, respectively. C and D: Automated MALDI MS spectra recorded at the elution time 80 min (∼ 21% ACN) and 300 min (∼ 21% ACN) of the 1D and 2D separation; IS marks the spiked internal [Glu1]-Fibrinopeptide B standard. E: MALDI MSMS spectrum of the enriched crosslink precursor (m/z = 1943.67). Y-type ion series of the peptide moiety (y_p) and the y- and x-type ion series of the RNA moiety (y_r and x_r) are indicated as well as the observed base and phosphate losses (A′, U′, C′, P_i). The entire sequence of the crosslinked peptide and the RNA moiety with the observed fragment ions is shown in the spectrum.

peptides (245–365 min). As indicated by the multichannel absorption at 220, 254, and 280 nm, the RNA-containing species were exclusively detected and sequenced in the elution profile of the enriched species, whereas at the corresponding ACN concentration in the first elution gradient, no dual absorption of the crosslinks was detectable (Figure 4D).

Enrichment of Peptide–RNA Oligonucleotide Crosslinks from UV-Irradiated Native U1 snRNPs and Reconstituted [15.5K-61K-U4atac snRNA] Complexes

We next compared our 2D setup with conventional 1D nano-reversed phase (RP) setup. Therefore, we used native U1

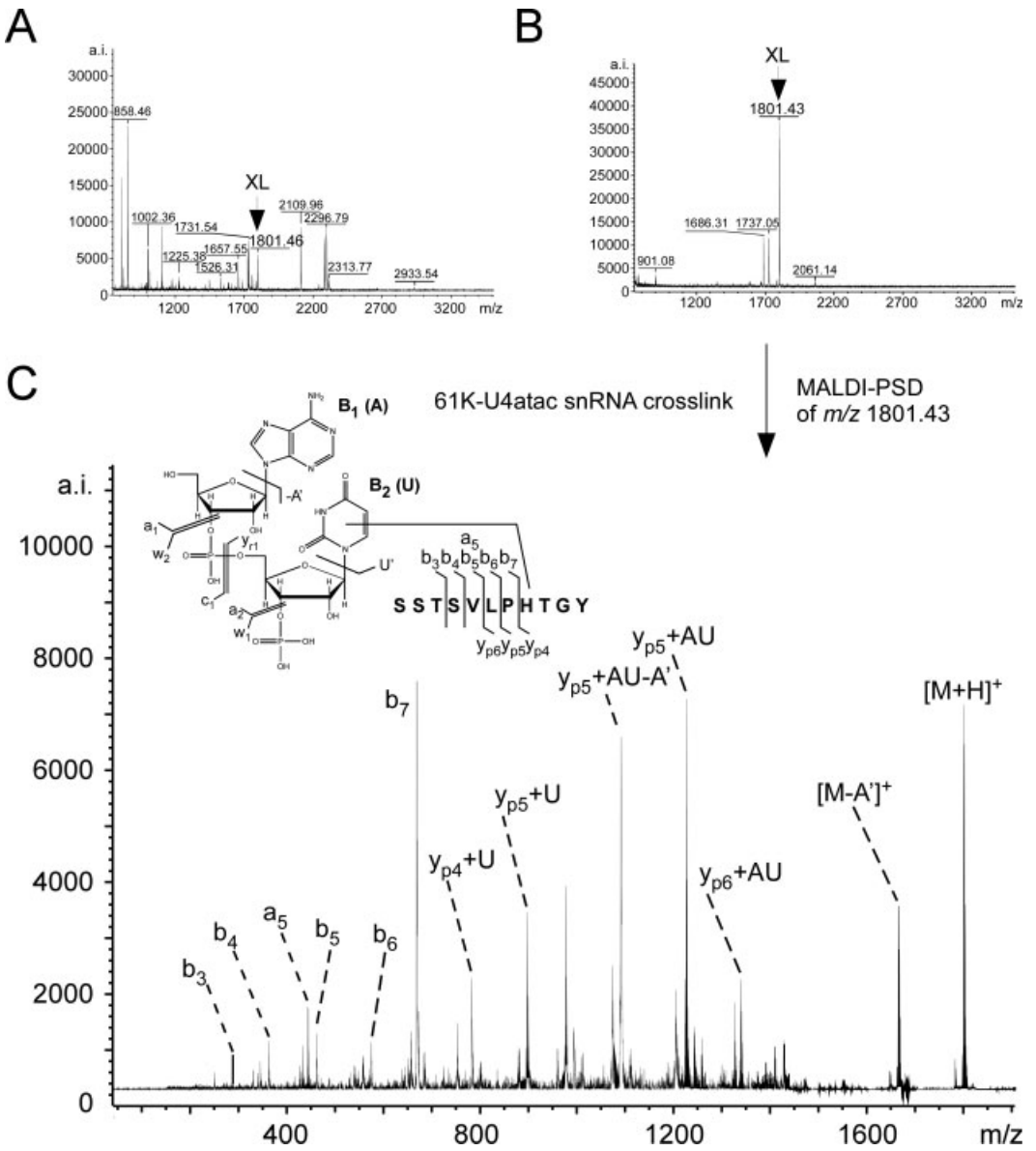

FIGURE 7 MALDI MS and MSMS analysis of enriched crosslinked peptide–RNA oligonuclotides derived from UV-irradiated U1 snRNP and [15.5K-61K-U4atac snRNA] hydrolyzed with RNases and endoproteinase chymotrypsin. A and B: MALDI MS spectra of 1D and 2D fraction at ~ 16% ACN containing putatively crosslinked species (m/z = 1801.43). C: MSMS (PSD) spectrum of the enriched precursor m/z = 1801.43. The spectrum reveals the crosslinked peptide sequenced derived from the 61K (hPrP31) protein crosslinked to an AU dinucleotide. Fragment ions of the crosslinked peptide and RNA sequence are depicted within the spectrum and the sequences. Y-type ion series of the peptide moiety (y_p) and the y- and x-type ion series of the RNA moiety (y_r and x_r) are indicated as well as the observed base and phosphate losses (A′, U′, C′, P_i).

snRNPs and reconstituted [15.5K-61K-U4atac snRNA] complexes and irradiated them at 254 nm according to Nottrott et al.[25,26] After dissociation of the complexes in the presence of urea, the RNA moiety was digested with RNase A, T1, and benzonase to generate RNA oligonucleotides that were as small as possible (see above). The protein moiety was hydrolyzed with trypsin or chymotrypsin, respectively, and the crude mixture was applied onto the 2D chromatographic system. In parallel experiments we injected the mixture onto a "standard" nano-LC system equipped with a precolumn for desalting of the sample, working in back-flush mode. Figures 5A and 5B show the elution profile of the standard 1D separation and the final elution step in the 2D enrichment, respectively, of completely hydrolyzed U1 snRNPs after UV irradiation. Figures 5C and 5D show the respective elution profiles from completely hydrolyzed [15.5K-61K-U4atac snRNA] after UV irradiation. As expected, the elution profile of the standard 1D gradient is much more complex as compared to the 2D separation. Nonetheless, by monitoring the absorbance at 214 and 254 nm, putative crosslinks were also detected at lower concentration of ACN (apparent at 21% or 16% ACN in the chromatogram). In the 2D elution profile these putative crosslinks represent almost the only peaks that were detectable, especially in the U1 snRNP sample.

We analyzed the spotted fractions of the both gradients (1D and 2D) by MALDI MS and MSMS for the enrichment of peptide–RNA crosslinks (Figures 6 and 7). Figures 6A and 6B show the 2D heat maps of the eluted species in the 1D and 2D separation of the crosslinked U1 snRNP, respectively. The complexity was dramatically reduced by the enrichment step. MALDI MS spectra were recorded at corresponding elution times ($\sim$ 21% ACN) containing the U1 70K specific peptide RVLVDVER crosslinked to a di or trinucleotide. The high complexity of the fraction before the enrichment did not reveal the putatively detectable peptide–RNA crosslinks. As the total sample amount per spot is not enough to select more than 10 precursors for extensive MALDI-MSMS analysis we could not obtain sequence information for all precursors present in the fraction. Consequently, the low-abundance crosslinks were not automatically selected and sequenced. In contrast, corresponding fractions in the 2D separation show significantly less complexity, and putative crosslinked peptide–RNA oligonucleotides derived from U1 snRNP complexes are clearly enriched (Figures 6C and 6D). The MALDI-MSMS spectrum from the precursor m/z = 1943.67 revealed sufficient sequence information on the crosslinked RNA and peptide moiety for unambiguous identification (Figure 6E). As expected from MALDI analysis, we observed a strong neutral loss (-98 a.m.u.) revealing a crosslinked RNA moiety. By annotation of the observed fragment ions, the sequence of the crosslinked peptide was deduced to be VDVER. The further detected loss of an adenine base (135 a.m.u.), cytosine base (112 a.m.u.), cytidine (305 a.m.u.), and adenosine (329 a.m.u.) confirm the crosslink to be the U1 70K peptide RVLVDVER, crosslinked to an AUC trinucleotide (Figure 6E). One other enriched precursor in the shown fraction (Figure 6D) was RVLVDVER crosslinked to AU (m/z = 1638.68).

Figures 7A and 7B show fractions of the 1D and 2D separation, respectively, of completely hydrolyzed UV-irradiated [15.5K-61K-U4atac snRNA] complexes eluting at $\sim$ 16%. In the corresponding 2D fraction, the putative crosslink at m/z = 1801.43 is clearly enriched. MSMS experiments identified this enriched species being a chymotryptic peptide derived from the U4-specific 61K (hPrp31) protein crosslinked to an AU dinucleotide (SSTSVLPHTGY to AU, Figure 7C).

CONCLUSIONS

We report here the efficient on-line capturing and recovery of peptide–RNA crosslinks after total hydrolysis of UV-irradiated protein–RNA complexes. For this purpose we introduced a novel 2D chromatographic approach using TiO_2 columns for the enrichment of the crosslinked species. In contrast to our previous studies, the set-up has enabled us to isolate peptide–RNA oligonucleotide conjugates directly from crosslinked and completely hydrolyzed RNP complexes without the need for time-consuming SE chromatography, ethanol precipitation, and stepwise digestion of the protein and RNA moieties. Moreover, the use of nanochromatographic equipment (i.e., fused silica with i.d. of 50 to 100 μm for analytical columns and with 150 to 300 μm i.d. for precolumns) reduced the sample amount that was loaded on the system and still allowed detection and sequencing of the crosslinked species down to 4 μg (12 pmol).

On-line coupling of TiO_2 columns into a HPLC system or integrated within a nanochip has recently been reported for the enrichment of phosphopeptides from mixtures.[18,19,21] Although comparable to our system, in that two C18 trapping columns were used for additional desalting steps before and after the enrichment procedure and thus nonphosphorylated peptides can be directly analyzed in the flow-through by MS, this particular setting cannot be used for the enrichment of peptide–RNA crosslinks from mixtures. Both of the systems described by these authors use a series connection (hence termed "sandwich" set-up) of C18 precolumn, TiO_2 column, and C18 precolumn for the three chromatic steps of desalting, enrichment, and desalting before the final separation of the enriched species. In contrast, the first desalting step in our case achieved the removal of the excess of non-

crosslinked RNA moiety. In a series connection the RNA moiety would be washed directly onto the TiO_2 column and thus would presumably drastically minimize the binding capacity of the TiO_2 as it strongly interacts with the column material trough the phosphate backbone of the RNA. Moreover, the RNA would be eluted onto the analytical column during the further chromatographic steps and would then drastically interfere with the detection of other species in the MS (F.R., H.U., data not shown). For these reasons we had to use a two-valve system (as is, for example, realized within the dual-gradient system from Dionex/LC packings company) in which two trapping columns and a TiO_2 could be mounted, loaded, and washed independently of each other. This enabled us to remove the excess of noncrosslinked RNA from the entire system.

In this feasibility study, we coupled the chromatography system via a MALDI target spotter to MALDI MS and MSMS analysis. At present we consider this to be advantageous for several reasons: (i) TFA can be used as an additive in loading, in washing and in both the separation steps. In general TFA yields better separations of the crosslinked species as compared with formic acid in RP-LC (H.U, unpublished data) owing to its strong ion-pairing properties, but it is still completely compatible with MALDI analysis, although not with ESI MS at suitable concentrations [i.e., $\geq$ 0.1% (v/v)].[28,29] (ii) We found that loading and washing conditions with 0.25% (v/v) TFA generate a pH value of 1.5, which is close to the pI of an organic phosphate group and is still suitable for RP-chromatography. Recently Jensen and Larsen[22] and Thingholm et al.[16] described an optimized protocol for the efficient enrichment of phosphopeptides by TiO_2 using highly acidic conditions (5% TFA) in combination with 200 mg/ml DHB. Unfortunately such conditions are not compatible with on-line TiO_2-LC separations. (iii) 2,5-DHB as MALDI matrix in combination with up to 1% phosphoric acid has been shown to increase dramatically the signal intensity in MS mode of phosphopeptides[30] and peptide–RNA crosslinks.[6] (iv) In general MALDI analysis of spotted fractions allows the fully automated MS and MSMS analysis of selected precursors.[31] An advantage over ESI in this case is that samples can be reinvestigated once an enriched precursor has been identified as potentially containing a crosslink (or as potentially being a phosphopeptide). Initial MSMS analysis in MALDI easily reveals the loss of phosphate, even in low-abundance samples in a first investigation. Upon reinvestigation of the same spot, the remaining amount of precursor can be used for more in-depth sequencing by MSMS.

In recent phosphoproteomics applications, bound species are eluted from TiO_2 by a shift from pH $<$ 1 (5% TFA, loading, and washing) to pH 10.5 (NH_4OH). We also tested NH_4OH in our on-line settings but observed that the TiO_2 columns (at least in our hands) did not withstand these strongly basic conditions, so that TiO_2 columns had to be changed after each separation cycle. We therefore used 75 m*M* $(NH_4)_2HPO_4$ pH 1.1 in water. $(NH_4)_2HPO_4$ allowed us to use higher PO_4-concentrations giving a lower pH value than obtained from the same concentration of *o*-phosphoric acid alone. In our previous studies we successfully applied phosphoric acid as a replacement for peptide–RNA oligonucleotides from IMAC beads.[12] In the work presented here, we were able to demonstrate that PO_4 also works for elution from TiO_2 and, importantly, that the TiO_2 column could be used up to 30 times without significant loss in its binding properties. Along the same lines, Jensen and Larsen[22] observed a compromising effect of phosphate ions in buffers for the binding of phosphopeptides, supporting our finding that inorganic phosphate is an applicable reagent for the elution of phosphate-containing species.

As described earlier, TFA as ion-pairing reagent interferes with ESI-MS. Future studies should therefore investigate whether alternative ESI compatibles additives (like formic acid or acetic acid, as used by Cantin et al.,[20] Pinkse et al.,[18] and Mohammed et al.[21]) are appropriate for loading and washing of TiO_2 columns for the enrichment of crosslinked species. We recently demonstrated that crosslinked species can be detected and sequenced by multiple reaction monitoring-triggered MSMS in an ESI hybrid linear ion trap/triple quadrupole MS in the positive mode using "standard" ESI-RP solvent, i.e., ACN and FA.[10] Coupling our 2D system to an ESI linear ion trap/triple quadrupole MS would enable a high-throughput analysis of different UV-irradiated protein–RNA complexes.

MATERIALS AND METHODS

Sample Preparation

Spliceosomal [15.5K-61k-U4 snRNA] and U1 snRNPs were reconstituted and purified as described elsewhere.[25,26] For crosslinking, 6.75 nmol (470 μg) and 152 pmol (50 μg) of the complexes, respectively, were UV-irradiated at 254 nm in a volume of 12 ml glass dishes (in-house) and 3 μl $\times$ 100 μl in low-adhesive, flat-bottomed microtiter plates (Greiner), respectively, for 3 min as described in Urlaub et al.[7,8] Irradiated samples are precipitated with ethanol, washed, and dried in a SpeedVac exactly as described in Kühn-Hölsken et al.[6] Samples were dissolved in 4*M* urea, 50 m*M* Tris-HCl pH 7.5. After dilution to 1*M* urea using 50 m*M* Tris-HCl pH 7.5, the RNA was digested by adding 1 μg RNase A, 1 μg RNase T1, and 125 U benzonase at 52°C for 2 h. Subsequently the protein moiety was digested by adding 7.5 μg trypsin (modified, Promega) (enzyme:substrate ratio 1:20) at 37°C overnight. [15.5K-61K-U4atac

snRNA] complexes were digested with 23.5 μg chymotrypsin (Roche) at 37°C over night.

The U1 70K peptide crosslink (RVLVDVER) was purified on a semipreparative scale from UV-irradiated U1 snRNPs exactly as described by Kühn-Hölsken et al.[6] Purified fractions were dried in a speed-vac and stored for further use.

Enrichment of Phosphopeptides and Crosslinks by Off-Line TiO_2 in Batch

A slurry from TiO_2 bulk material (5 mg/ml) in loading buffer 0.25% (v/v) TFA, 50% (v/v) ACN was prepared and 20 μl aliquots (equivalent to 0.1 μg) were used for batch enrichment. Sample was loaded in 50 μl loading buffer by vigorous shaking for 15 min. Then TiO_2 particles were sedimented at 13,000 rpm and the supernatant was collected. Washing was performed with two increasing concentrations of ACN [25% (v/v) and 50% (v/v)] again incubating for 15 min. Finally, the sample was eluted twice with 50 μl $(NH_4)_2HPO_4$ pH 1.1 buffer and phosphorylated and crosslinked peptides were recovered onto stage tips (C18 3M Empore Disk incorporated into gel loader tips). All steps were monitored by preparing equivalent amounts of sample (0.5 μl from 50 μl) onto Bruker Anchor MALDI plates using 2,5-DHB matrix.

1D/2D Chromatography Setup

The on-line chromatography system (1D and 2D) was mounted into an Ultimate Dual Gradient System (Dionex, Idstein, Germany) equipped with an autosampler (Sparck, Emmen, Netherlands), and a multichannel UV detector (Dionex) with a 3 nl flow cell. Analytical columns were prepared in fused silica capillaries (PT Polymicro Technologies L.L.C.) with 75 μm i.d. and 375 μm o.d that had been capped on one side by a Kasil™ (PQ Europe) frit of 2–5 mm length. Analytical columns were packed by using a pressure vessel for column packing (Bruchbühler, CH) using Vydac 218TP5215 material (particle size 5 μm, pore width 300 Å; Vydac, Columbia, molecular dynamics (MD), USA) to a length of ~ 15 cm. Precolumns were prepared from either ReproSil-Pur Basic C18-HD (5 μm particle size, 120 Å pore width, Dr. Maisch GmbH, Ammerbuch, Germany) or Vydac 218TP5215 material in the same manner by using fused silica capillaries with 150 μm i.d. and 375 μm o.d. Precolumns were trimmed to a length of 2.5 cm and capped at both ends with Micro-Tight Fittings with 0.5-μm peek filter end fittings (UpChurch Scientific, Oak Harbor, WA). Titanium dioxide enrichment columns with 100 μm i.d. were prepared in the same manner using 5 μm titanium dioxide beads (Sigma Aldrich) that had been washed free from smaller debris.

Connecting capillaries had an i.d. of 25 μm for standard 1D chromatography and 50 μm for 2D chromatography set-up. The loading pump was operated at a flow rate of 9 μl/min (1D) and 5 μl/min (2D) with loading solvent [3.5% (v/v) ACN/0.1% (v/v) TFA]. The nanopump was run at 300 μl/min, generating a gradient from 10 to 60% solvent B_1. Solvents were as follows: for standard 1D LC, solvent A was 0.1% (v/v) TFA in water and solvent B was 80% (v/v) ACN/0.1% (v/v) TFA in water; for 2D chromatography solvent A_1 and A_2 were 0.25% (v/v) TFA and solvents B_1 and B_2 were 80% (v/v) ACN/0.25% (v/v) TFA. The capillary pump operated at a flow rate of 3.5 μl/min with solvents A_2 and B_2 or with solvent C for elution (10 mg/ml (75 m*M*) $(NH_4)_2HPO_4$ pH 1.1).

1D/2D Workflow

For 1D separation, samples were loaded for 15 min onto the precolumn and eluted by backflush on the analytical column. Separation was carried out for 120 min. The analytical column was washed with 80% (v/v) ACN, 0.1% TFA for 9 min, and re-equilibrated under starting conditions [10% ACN (v/v)].

The column configurations of the 2D set-up are illustrated in Figure 3. The initial 10-port valve configuration was 10-1/1-2. The sample was loaded for 25 min onto the precolumn. During loading in this configuration the TiO_2 column was equilibrated with 48% ACN, 0.25% (v/v) [60% (v/v) solvent B] TFA in water using the capillary pump at a flow of 3.5 μl and the second precolumn was equilibrated at 300 nl/min with 8% ACN (v/v), 0.25% TFA (v/v) using the nanopump. By switching the two 10-port valves simultaneously, 1-2 and 10-1, the samples were eluted from the precolumn to the TiO_2 column and to the analytical column by applying a gradient of 0 to 48% ACN (v/v), 0.25% TFA for 120 min via the nanopump. By switching the valves to positions 1-2 and 1-2, bound species on the TiO_2 column were eluted with 75 m*M* $(NH_4)_2HPO_4$ pH 1.1 for 35 min at a flow rate of 3.5 μl onto the second precolumn (ReproSil-Pur Basic C18-HD). The precolumn was flushed with 2.5% TFA in water for 30 min. During this time the analytical column was equilibrated with 0.25% TFA (v/v) (100% solvent A_1) at a flow rate of 300 nl/min by the nanopump. Elution of the bound species from the precolumn is achieved by switching the valves to positions 10-1 and 1-2 and applying a gradient of 0 to 48% ACN (v/v), 0.25% TFA (v/v) (0 to 60% solvent B_1) in TFA water for 120 min.

MALDI Mass Spectrometry

The eluate in 1D or in 2D chromatography from the first and second gradient was mixed in a 29-nl T-piece (UpChurch Scientific, Oak Harbor, WA) with either α-cyano-4-hydroxy cinnamic acid [HCCA, 10 mg/ml in 70% (v/v) ACN/0.1% (v/v) TFA] containing 10 fmol/μl Glu-fibrinogen peptide (*m/z* 1570, Sigma) as internal standard (1D standard LC) or with 2,5-dihydroxybenzoic acid [DHB, 10 mg/ml in 20% (v/v) ACN/0.1% (v/v) H_3PO_4 containing an internal standard Glu-fibrinogen peptide], respectively, delivered at a flow rate of 0.9 μl/min. Fractions were spotted every 15 s (α-cyano) onto LC-MALDI stainless steel plates or every 30 s (DHB) onto 384er stainless steel MALDI targets (Applied Biosystems/Sciex MDS, Foster City) and 384er Bruker Anchor600 chip plates (Bruker-Daltonics, Bremen, Germany) by a Probot Spotter (Dionex, Idstein Germany). MS analysis was performed either on a MALDI-ToF/ToF 4800 proteome analyzer (Applied Biosystems/Sciex MDS, Foster City) or a Bruker Reflex IV instrument (Bruker-Daltonics, Bremen, Germany). For MS spectra in positive-ion mode on an ABI 4800 ToF/ToF analyzer, a total of 800 shots were generated and for MSMS a maximum of 5000 shots were accumulated for each precursor, using dynamic stop criteria depending on the spectral quality. Job-wide interpretation of the 4800 ToF/ToF analyzer generated MS data allowed the 15 highest peptides in intensity for sequencing in MSMS mode. Collision energy was set to 1×10^{-6} torr, with potential difference between the source acceleration voltage and the collision cell set at 1 kV. For MS spectra in positive-ion mode on a Bruker Ultraflex IV instrument, 200 shots were summed up. If a suitable high amount of precursor was detected, one MALDI-PSD spectrum from a spot could be detected. PSD spectra were recorded with an acceleration voltage of 25 kV (IS1)

and 20.2 kV (IS2). The delay time was 200 ns, and a total of 210 laser shots were summed for each PSD segment.

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